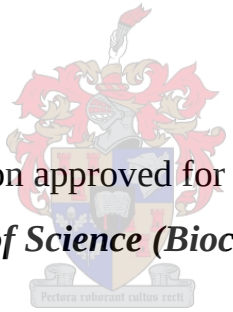
Production, characterisation and activity of selected and novel antibiotic peptides from soil bacteria

by

Wikus Ernst Laubscher
B.Sc. Honours (Biochemistry)

December 2016



Dissertation approved for the degree
Masters of Science (Biochemistry)
in the
Faculty of Science
at the
University of Stellenbosch

Supervisor: Prof. Marina Rautenbach
Department of Biochemistry
University of Stellenbosch

Declaration

I, ***Wikus Ernst Laubscher*** hereby declare that the entirety of the work contained in this thesis is my own original work and that I have not previously in its entirety or in part submitted it for obtaining any qualification at any university.

…… 23/05/2016 ……

Wikus Ernst Laubscher Date

Summary

The ever increasing development of pathogen resistance towards conventional antibiotics has necessitated the search for novel antimicrobial molecules. It has been suggested that antimicrobial peptides will form the foundation of a new generation of antibiotics. These small natural antibiotics possess rapid killing mechanisms against a broad spectrum of pathogens. They often disrupt multiple cellular targets resulting in decreased risk of resistance, making them ideal candidates as novel antimicrobials. Selection of a screening source is challenging as antimicrobial peptides are ubiquitously produced by most organisms. However, soil bacteria have historically been shown to produce a large variety of clinically significant antibiotics, including antimicrobial peptides. Furthermore, the soil biome possesses a vast bacterial diversity that has been to date largely unexplored, making it an ideal resource for the discovery of novel antimicrobial peptides. The overall objective of this study was therefore to isolate, identify and characterise novel antimicrobial peptides from soil bacteria.

A commercial soil additive containing a lower bacterial diversity than natural soil environments was first used to validate a method for novel antimicrobial peptide discovery. With the goal of discovering novel antimicrobial peptides, this method included the isolation, identification and characterisation of antimicrobial producing bacteria and their active components. Only two of the isolates from the soil additive, denoted LB.4 and LB.5, were selected for further purification and characterisation of their antimicrobial compounds. The LB.4 and LB.5 isolates were determined to be strains of *Brevibacillus laterosporus* and *Bacillus licheniformis* respectively. In this dissertation they are referred to as *Br. laterosporus* LB.4 and *B. licheniformis* LB.5. The antimicrobial compound produced by *B. licheniformis* LB.5 was determined to be the well–known antimicrobial peptide, bacitracin A. It was found that *Br. laterosporus* LB.4 produces two potentially novel antimicrobial peptides termed LB.4-1223 and LB.4-1273. Although the amino acid composition was shown to be: F, L/I, M, N, P, V and Y, the amino acid sequences remain to be determined and their novelty could therefore not be confirmed. *Brevibacillus parabrevis* was used as a positive control as it is known to produce antimicrobial peptides from the tyrocidine group. The biological activity and mode of action data of the isolated peptides were therefore compared to that of tryptocidine C, a characterised analogue from the tyrocidine group of peptides. Antimicrobial dose response analysis revealed that LB.4-1223, LB.4-1273 and tyrocidine C possess

antimicrobial and haemolytic activity, while bacitracin A only showed potent antimicrobial activity.

Biophysical studies indicated that both bacitracin A and tryptocidine C disorientated lipid bilayers, however, only tryptocidine C resulted in the formation of transmembrane pores. This was the first study to show pore formation by tryptocidine C. It is known that bacitracin elicits its antimicrobial activity by inhibiting peptidoglycan synthesis, but this study provided the first insight into the interactions between bacitracin A and cellular membranes. However, whether these interactions results in microbial inhibition is still unknown.

Screening of an environmental soil sample yielded three isolates with significant antimicrobial activity and suitable low molecular mass spectra to suggest antimicrobial peptide production. A literature study suggested that one of the isolates produces peptides from the bogorol group of antimicrobial peptides, while the other two isolates produce previously uncharacterised compounds. These compounds will be investigated in future studies.

This dissertation describes an antimicrobial discovery and characterisation study that led to the discovery of novel antimicrobial peptides/compounds. Furthermore, it was shown that the laborious, time consuming nature of traditional microbiological screening methodology demands that a more effective, higher-throughput methodology be developed to meet the demand for novel antibiotics discovery.

Opsomming

Die onbeheerde toename in patogeen-weerstand teenoor konvensionele antibiotika het die soektog na nuwe antibiotiese molekules genoodsaak. Daar word gespekuleer dat antimikrobiese peptiede die basis van ŉ nuwe generasie antibiotika sal vorm. Hierdie klein, natuurlik geproduseerde antibiotika besit vinnige uitwissingsmeganismes teen a breë spektrum patogene. Antimikrobiese peptiede onderdruk dikwels veelvuldige sellulêre teikens wat lei tot ŉ verlaagde weerstandigheidsrisiko, wat hul dus ideale kandidate maak vir nuwe antimikrobiese terapieë. Meeste organismes produseer antimikrobiese peptiede wat die keuse van siftingsmateriaal bemoeilik. Histories is dit wel duidelik dat bakterieë afkomstig van grond ŉ groot verskeidenheid klinies-belangrike antibiotika produseer, insluitend antimikrobiese peptiede. Die grond bioom besit ook ŉ enorme bakteriële diversiteit wat nog grootliks onverkend is en is dus ŉ ideale hulpbron in die soektog vir nuwe antimikrobiese peptiede. Daarom is die hoofdoel van hierdie studie om nuwe antimikrobiese peptiede afkomende van grond bakterieë te isoleer, identifiseer en te karakteriseer.

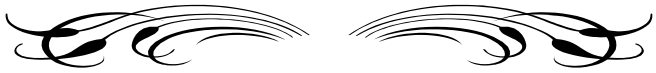
Eerstens is ŉ kommersiële grondbymiddel, wat ŉ laer verskeidenheid van bakteriële spesies in vergelyking met natuurlike grond omgewings bevat, gebruik om ŉ geldige metodiek te ontwikkel vir die ontdekking van nuwe antimikrobiese peptiede. Isolasie, identifikasie en karakterisering van antimikrobiese bakterieë en hul antimikrobiese aktiewe molekules is ingesluit in hierdie metodiek met die doel van nuwe antimikrobiese peptied ontdekking. Net twee van die grondbymiddel-isolate, genoem LB.4 en LB.5, is geselekteer vir verdere suiwering en karakterisering van hul antimikrobiese komponente. Die LB.4 en LB.5 isolate was as *Brevibacillus laterosporus* en *Bacillus licheniformis* geïdentifiseer en word na *Br. laterosporus* LB.4 en *B. licheniformis* LB.5 verwys in hierdie skripsie. Daar is beplaal dat die antimikrobiese molekule wat deur *B. licheniformis* LB.5 geproduseer word die wel-bekende antimikrobiese peptied basitrasien A is. Daar is verder gevind dat twee, moontlike nuwe, antimikrobiese peptiede geproduseer word deur *Br. laterosporus* LB.4, genoem LB-1223 en LB-1273. Die peptiede se aminosuursamestelling was vasgestel as F, L/I, M, N, P, V en Y, tog kon die uniekheid van die nuwe peptiede nog nie bevestig word nie weens die onbekende aminosuurvolgorde, wat eers in toekomstige studies ondersoek sal word. *Brevibacillus parabrevis* is bekend vir die produksie van die antimikrobiese peptiede van die tirosidien groep. Inligting van biologiese aktiwiteit en meganisme van werking studies op die geïsoleerde peptide is vergelyk met die gekarakteriseerde tirosidien analoog, triptosidien C.

Antimikrobiese dosisrespons analise het bevestig dat LB.4-1223, LB.4-1273 en triptosidien C beide antimikrobiese en hemolitiese aktiwiteit besit, terwyl basitrasien A het egter net kragtige antimikrobiese aktiwiteit getoon het.

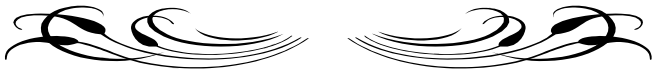
ʼn Biofisiese ondersoek het aangedui dat beide basitrasien A en triptosidien C dubbellaag membrane disoriënteer, maar dat net triptosidien C transmembraanporieë vorm. Dit was die eerste bewys dat triptosidien C porieë in lipiedmembrane vorm. Dit word algemeen aanvaar dat basitrasien se hoofmeganisme van werking die inhibisie van peptidoglikaansintese behels. In hierdie studie is die eerste insigte verskaf tot die interaksie van basitrasien A met dubbellaagmembrane. Dis nog onbekend of hierdie membraan interaksie wel ʼn rol in mikrobiese inhibisie het.

In die ondersoek op ʼn omgewingsgrondmonster is net drie isolate gevind met betekenisvolle antimikrobiese aktiwiteit en lae molekulêre massa spektra wat dui op moontlike antimikrobiese peptiede produksie. Uit literatuurstudies het dit geblyk dat een van die isolate peptiede vanuit die bogorol groep van antimikrobiese peptiede produseer, terwyl die oorblywende isolate ongekarakteriseerde verbindings/peptide produseer. Die bogenoemde verbindings/peptiede sal verder ondersoek word in toekomstige studies.

Hierdie tesis beskryf ʼn antimikrobiese ontdekkings- en karakteriseringstudie wat gelei het tot die ontdekking van nuwe antimikrobiese verbindings/peptiede. Verder beklemtoon die studie die moeisame, tydrowende aard van tradisionele mikrobiese siftingsmetodologie en sodoende die noodsaak vir nuwe, hoër-deurset metodologie om aan die aanvraag na nuwe antibiotka te voldoen.



"*Knowledge is limited. Imagination encircles the world*." - Albert Einstein



Acknowledgements

I would like to express my thanks to the following people and institutions:

- Prof. Marina Rautenbach, my supervisor, for all her encouragement, sympathy, motivation, support and guidance throughout this thesis, as well as allowing my imagination to run wild.
- Dr. Marietjie Stander and all the staff at the Central Analytical Facility (CAF) at the University of Stellenbosch for their assistance in mass spectrometry analysis.
- Prof. Burkhard Bechinger and Dr. Christopher Aisenbrey at the University of Strasbourg for being warm, welcoming hosts during a research trip to France as well as their assistance and guidance during NMR experiments.
- Prof Jan C. Behrends, Dr. Ekaterina Zaitseva and the members of their group at the University of Freiburg for doing the electrophysiological experiments and the very nice lunch.
- Ms. Helba Bredell for her support, nice midmorning chats and mostly for her immense efforts in making our laboratory run smoothly.
- My colleagues at the BIOPEP peptide group for their support, companionship and making every day in the lab a day to remember.
- Dr. Nic Lombard for his innovative ideas and stimulating conversations.
- My parents for their immense love, belief, patience, emotional and financial support, you have inspired me to always believe in my dreams.
- Meghan Perkins for her love, sympathy, support both emotionally and academically, without you my life would just be a little bit darker.
- My brothers and sister for always reminding me to have a little fun and for making family gatherings memorable.
- All my friends who have been wondering where I've been hiding during this thesis for always being there and providing some much needed sympathy and the occasional braai.
- Harry Crossley Foundation, University of Stellenbosch and the BIOPEP peptide group providing the funds for me to expand my scientific knowledge and finish this degree.

Table of Contents

CHAPTER 4

CHAPTER 5

List of Abbreviations and Acronyms

$[M+2H]^{2+}$	doubly charged molecular ion
$[M+3H]^{3+}$	triply charged molecular ion
$[M+H]^{+}$	singly charged molecular ion
^{31}P	phosphorus-31
ACN	acetonitrile
ATCC	American type culture collection
B. licheniformis	*Bacillus licheniformis*
B. subtilis	*Bacillus subtilis*
BMAP-23	bovine myeloid antimicrobial peptide 28
Br. laterosporus	*Brevibacillus laterosporus*
Br. parabrevis	*Brevibacillus parabrevis*
BT-NRPS	*Brevibacillus texasporus* non-ribosomal peptide synthetase
C. albicans	*Candida albicans*
CoA	coenzyme A
CXCR4	chemokine receptor type 4
Da	Dalton
dlt	D-alanyl-lipoteichoic acid
E. coli	*Escherichia coli*
E. terrae	*Eleftheria terrae*
EDTA	ethylenediaminetetraacetic acid
ESMS	electrospray mass spectrometry
ESMS/MS	electrospray tandem mass spectrometry
f	D-phenylalanine
F	L-phenylalanine
GRAS	generally recognized as safe
HC_{50}	peptide concentration leading to 50% haemolysis
HC_{max}	peptide concentration leading to maximum haemolysis
HEPES	hydrozyethyl piperazineethanesulfonic acid
HIV	human immunodeficiency virus
HIV-1	human immunodeficiency virus type 1
HPLC	high performance liquid chromatography
HSV	Herpes simplex virus
IC_{50}	peptide concentration leading to 50% microbial growth inhibition
IC_{max}	peptide concentration leading to maximum microbial growth inhibition
K	Kelvin
L	leucine

LAB	lactic acid bacteria
LB	Luria Bretani
LPG	lysylphosphatidylglycerol
M. luteus	*Micrococcus luteus*
m/z	mass over charge ratio
MALDI	matrix-assisted laser desorption ionization
MDR	multidrug-resistant
MECA	micro electrode cavity array
MIC	minimum inhibitory concentration
mprF	multiple peptide resistance factor
M_r	molecular mass
MRSA	methicillin resistant *Staphylococcus aureus*
MS	mass spectrometry
MS_2	second mass spectrometric analyser
N	asparagine
n	number of biological repeats
NB	nutrient broth
NCBI	national center for biotechnology information
NCTC	national collection of type cultures
NMR	nuclear magnetic resonance
NRPS	non-ribosomal peptide synthetase
O	ornithine
OD	optical density
Orn	ornithine
P	proline
P. acnes	*Propionibacterium acnes*
P. digitatum	*Penicillium digitatum*
P. falciparum	*Plasmodium falciparum*
pA	picoampere
PBS	phosphate buffered saline
PCP	peptidyl carrier protein
PCR	polymerase chain reaction
Pn-AMP1	*Pharbitis nil* antimicrobial peptide 1
POPC	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine
POPE	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine
POPG	1-palmitoyl-2-oleoyl-sn-glycero-3-phosphoglycerol
PP	phosphopantetheine
ppm	parts per million
Q	glutamine

ROS	reactive oxygen species
RP-HPLC	reverse phase high performance liquid chromatography
RPMI	Roswell park memorial institute medium
rRNA	ribosomal ribonucleic acid
RsAFP2	*Raphanus sativus* antifungal peptide 2
S. aureus	*Staphylococcus aureus*
SD	standard deviation
SEM	standard error of the mean
sp.	specie
spp.	species in plural
Te	thioesterase domain
TFA	trifluoroacetic acid
TGS	tryptone, glucose and salt growth medium
TLC	thin layer chromatography
TOF	time of flight
Tpc A	tryptocidine A
Tpc B	tryptocidine B
Tpc C	tryptocidine C
Tpc C_1	tryptocidine C_1
Trc A	tyrocidine A
Trc B	tyrocidine B
Trc C	tyrocidine C
Tris	tris(hydroxymethyl)aminomethane
Trp	tryptophan
TSB	tryptone soy broth
UDP	uridine diphosphate
UPLC	ultra-performance liquid chromatography
V	valine
v/v	volume/volume
VSV	vesicular stomatitis virus
w	D-tryptophan
W	L-tryptophan
Y	tyrosine
YadA	*Yersinia* adhesion A domain
α-CHCA	α-cyano-4-hydroxycinnamic acid

Preface

It has been well-established that conventional antibiotics are becoming increasingly ineffective due to pathogenic organisms developing antibiotic resistance. This has led to prolonged illness and increased morbidity and mortality rates. Aside from the medical implications, antibiotic resistance also has alarming economic impacts. The World Health Organisation has estimated that in the USA alone, the economic effect of antibiotic resistance on the health system per year is around 21 to 34 billion dollars.

Some of the first antimicrobial therapeutics discovered included antimicrobial peptides. However, the generally higher antimicrobial activity and lower cytotoxicity of conventional antibiotics resulted in the underdevelopment of antimicrobial peptides as clinical therapeutics. Only recently, antimicrobial peptides are being revived as a promising solution to replace or substitute conventional antibiotics. This is due to the rapid antimicrobial activity, as well as lower potential of pathogen resistance development of these small naturally produced antimicrobial agents. Although antimicrobial peptides are ubiquitously produced by most organisms, it has been demonstrated that soil bacteria are one of the most enriched sources of antibiotics including antimicrobial peptides. Hundreds of antimicrobial peptides from various soil bacteria have already been isolated and characterised, many of which play a crucial role in various practices such as culinary preservatives, last resort antibiotics and topical disinfectants.

The goal of this study was therefore to discover novel antimicrobial peptides, specifically from soil bacteria, so as to aid in the current battle against antibiotic resistant pathogens. To obtain this goal the following objectives were set:

- Isolation of bacteria producing antimicrobial compounds from a low diversity source (commercial soil additive) to validate and develop necessary screening methodology and possibly identify novel antimicrobial peptide(s).
- Screening for distinct bacterial isolates from a soil additive that produce novel antimicrobial peptide(s)/compound(s) using traditional microbiological methods (Chapter 2).
- Development and use of a rapid method utilising mass spectrometry to determine whether antimicrobial peptides might be present and if they have been previously characterised (Chapter 2).
- Purification and identification of antimicrobial peptide(s)/compound(s) produced by selected bacterial isolates from the soil additive (Chapter 3).
- Characterisation of antimicrobial activity, haemolytic activity and selected biophysical properties of purified antimicrobial peptide(s)/compound(s) (Chapter 3)
- Selection of bacteria producing antimicrobial peptide(s)/compound(s) from an environmental soil sample using techniques validated in Chapters 2 and 3, to identify novel antimicrobial peptide(s)/compound(s) for future studies (Chapter 4).

In Chapter 1, we provide an overview of antimicrobial peptides, summarizing several of their general characteristics. Furthermore, this chapter includes a brief discussion on antimicrobial peptides produced from soil bacteria and explains why soil bacteria remain an invaluable source of novel antimicrobial therapeutics. The subsequent three experimental chapters report on the objectives described above, providing results and full discussions. In the final chapter (Chapter 5), the overall outcome of this dissertation is discussed and recommendations for future investigations are presented. Experimental chapters were written in the most part in the form of independent units. As such, repetition was inevitable, however, all attempts to avoid repetition were made where possible.

Outputs of MSc study

Conferences/Oral presentations

Laubscher, W.E. (2015) Production, characterization and activity of selected and novel antimicrobial peptides from soil bacteria. Biochemistry forum, Stellenbosch University, Stellenbosch, South Africa

Laubscher, W.E. (2015) Medium throughput assay for the detection and identification of novel antimicrobial peptides from soil bacteria, Antimicrobial peptides and biomaterials workshop, Strasbourg, France

Laubscher, W.E. (2016) Production, characterization and activity of selected and novel antimicrobial peptides from soil bacteria. Biochemistry MSc defense, Stellenbosch University, Stellenbosch, South Africa

Expected future outputs

Patenting of new peptides from this study will be done when the novelty of each has been confirmed.

Laubscher W.E., Rautenbach M. (2016) Purification, identification and characterisation of novel antimicrobial peptides from a bacterial strain isolated from a soil additive, article in preparation for submission to Microbiology.

Laubscher W.E., Rautenbach M. (2016) Discovery and characterisation of novel antimicrobial peptides from a bacterial strain isolated from a soil sample, article in preparation for submission to Applied and Environmental Microbiology.

Chapter 1
A broad overview of antimicrobial peptides

1.1 Introduction

The ever increasing development of resistance by pathogens towards conventional antibiotics has encouraged the search for new and unique antimicrobial molecules. Amongst naturally produced antimicrobial compounds, numerous investigators have predicted that antimicrobial peptides will form the foundation of a new class of antibiotics that is less susceptible to pathogen resistance [1-5].

Antimicrobial peptides are ubiquitously produced by most living organisms and exert a wide variety of activity against microbial organisms. These peptides often have several cellular targets and can even elicit their antimicrobial activity through multiple modes of action. This makes it more complex and uncommon for a microbe to develop antimicrobial resistance against these peptides [6-9]. However, their most important mode of action is their ability to selectively disrupt anionic cellular membranes [9,10].

The development of antimicrobial peptides as novel antibiotics has become increasingly attractive to researchers due to their rapid killing mechanisms and low susceptibility to antimicrobial resistance [6]. Although hundreds of antimicrobial peptides have already been identified, few have succeeded in clinical trials and been implemented in the pharmacological sector [11]. With the development of new and innovative technologies, it may be possible to enlarge the library of known antimicrobial peptides, possibly leading to further implementation of novel antimicrobial peptides in both the clinical and agricultural sectors.

In this literature review, a summary is given of the basic properties of antimicrobial peptides including their biosynthesis, mode of action and the mechanisms of resistance developed by pathogens. Furthermore, we will discuss the practical applicability of antimicrobial peptides.

Lastly, we give a short overview of antimicrobial peptides from soil bacteria specifically and discuss why soil is a rich environment for novel antimicrobial peptide discovery.

1.2 Antimicrobial peptides

Antimicrobial peptides are typically defined as small (less than 100 amino acid residues), cationic, amphipathic proteins that are characterised by their microbicidal activity against various microorganisms. These peptides have been identified in almost all life forms including bacteria, fungi, plants, invertebrates and vertebrates [12-16]. They form part of a primitive, yet effective, nonspecific innate immune system, which is the main immune defence mechanism in the majority of organisms [7,17]. The primary role of these peptides is to kill or inhibit the growth of competing or invading microorganisms or pathogens. It has been shown that, collectively, antimicrobial peptides have activity towards bacteria, fungi, parasites, viruses and even malignant cells [18-20]. Furthermore, antimicrobial peptides in vertebrates have been shown to stimulate and support essential elements of the innate- and acquired immune systems [17].

Since the discovery of the first commercially used antimicrobial peptide complex, tyrothricin, in 1929, more than 2600 antimicrobial peptides have been identified [21,22]. These peptides vary considerably in terms of their sequence and structure and are therefore difficult to categorize. However, antimicrobial peptides can be divided into four main structural groups namely: β-sheet peptides, α-helix peptides, extended peptides and loop peptides [1,17]. Additionally, antimicrobial peptides can also be divided into two broad groups according to their biosynthesis; non-ribosomally and ribosomally (or genetic encoded) produced antimicrobial peptides [19].

1.2.1 Biosynthesis

1.2.1.1 Non-ribosomal peptide synthesis

Non-ribosomally synthesised peptides are not gene-encoded and are predominantly produced by bacteria and fungi [23]. Numerous non-ribosomally synthesised peptides have been described thus far with a large variety in structures and activity including: antibiotics, immunosuppressive agents, cytostatic agents and siderophores [24-28]. Furthermore, these peptides may also possess several structural features that are not present in ribosomally synthesised peptides, such as non-proteinogenic amino acids, macrocyclisation, branched macrocyclisation, N-methylation, glycosylation and fatty acid incorporation among others [13,29]. These peptides are synthesised by large multi-enzyme complexes, collectively called non-ribosomal peptide synthetases (NRPS), which function as templates and possess the required biosynthetic machinery [13]. NRPS's have a multi-modular configuration where each module possesses the ability to add a specific amino acid to a peptide chain [30]. Furthermore, each module can be subdivided into domains, where each domain represents an enzymatic unit of the individual steps of the peptide synthesis process [31].

There are three essential domains in every module that are required for the synthesis of the peptide backbone. These domains are required for (1) substrate recognition and activation, (2) transport to the catalytic centres and (3) the formation of the peptide bonds [13]. The first of the above mentioned essential domains, the adenylation-domain (A-domain), is responsible for the selection and controlled entry of substrates into the synthesis process and the simultaneous ATP-dependent activation of the amino acids as aminoacyl adenylates [32]. Studies have shown that ten residues in the A-domain are responsible for substrate specificity, and can therefore be seen as the 'codons' of the non-ribosomal peptide synthesis process [13,33]. The discovery of the specificity of the A-domain has consequently led to the formation of a non-ribosomal code that is ever expanding as new findings are presented [34].

The second essential step in the process is the transfer of the activated amino acids to the cofactor in the thiolation-domain (T-domain) which is a peptidyl carrier protein (PCP) [35]. PCP is essentially the transferring unit of a module, however, the cofactor namely 4'-phosphopantetheine (4'PP) is vital for transfer of activated amino acids [36]. The inactive *apo*-PCP is post-translationally activated to its *holo*-form, HS-4'PP-PCP, by the transfer of 4'PP from coenzyme A (CoA) to a serine on PCP, catalysed by associated 4'-phosphopantetheinyl transferases in a process called priming [36,37]. However, most of the CoA present in cells is in the form of acyl-CoA and the binding of the acyl-CoA molecules to the PCP serines will lead to mispriming. This obstacle is overcome by the hydrolysis of the acyl groups, catalysed by the NRPS associated thioesterases type II [38]. After the activation of PCP, amino acids and peptides are covalently bond to the thiol group on 4'PP as thioesters [35].

The third and final step in the non-ribosomal synthesis process is the formation of the peptide bond, which is achieved by the condensation-domain (C-domain) of the module [39]. Here the nucleophile, aminoacyl-S-4'PP-PCP, is transferred to the electrophile, peptidyl-S-4'PP-PCP, of an adjacent module and a peptide bond is formed.

The directed peptide synthesis along the NRPS template is explained by a model proposed by Stein *et al.* [30], known as the multiple carrier thiotemplate model. In this model the domains of each module are organized as C-A-PCP [30]. This arrangement is referred to as the elongation module where the initiation module does not usually possess a C-domain [37]. The elongation of the peptides is governed by an affinity-mediated process, where the affinity of the A-domain towards HS-4'PP is at a maximum at the unloaded state and therefore the aminoacyl-S-4'PP remains in the acceptor position in the C-domain [40]. After the peptide bond is formed, however, the affinity of the peptidyl-S-4'PP is at its maximum towards the donor position on the C-terminal of the next module [40]. Thus to conclude, after expression

of NRPS-genes, PCP is in its *apo*-form which is activated by priming. Hereafter, the active *holo*-PCP is loaded with substrates via the A-domain which is followed by the condensation reaction of the C-domain to form a peptide bond. This process is repeated until the last module called the termination module where many of the linear peptides undergo macrocyclisation catalysed by type I thioesterase-domain (Te-domain) after which the peptide is released [41].

1.2.1.2 Ribosomal peptide synthesis

A vast majority of antimicrobial peptides are gene-encoded and therefore ribosomally synthesised [42]. These molecules are found in organisms from all three domains of life with a vast amount of variation in their sequence and structure [42-44]. Unlike the non-ribosomally synthesised peptides, the primary sequence of these molecules can only consist of the canonical 20 proteinogenic amino acids [45]. This method of synthesis therefore limits the structural diversity of the produced peptides to a certain degree. However, it has been shown that various post- or co-translational mechanisms do exist, providing these peptides with some degree of modifications. Some of these modifications result in peptides that bear a resemblance to the unique structures of non-ribosomally synthesised peptides such as macrocyclisation and the incorporation of various side chains [46].

Almost all ribosomally synthesised peptides are synthesised as a larger precursor peptide that is encoded by a structural gene [45]. The core peptide is then post-translationally modified by various mechanisms such as cyclization, proteolytic processing, glycosylation, amino acid isomerization etc. to form a modified core peptide [43,45]. Subsequently, the peptide is proteolytically cleaved into the active peptide, referred to as the mature peptide (Figure 1.1) [43,45]. The precursor peptide possesses an N-terminal leader sequence which is important for the recognition of the peptide by the post-translational modification enzymes [47]. Although rare, some precursor peptides, like bottromycins, do not possess a leader sequence

at the N-terminal but rather a follower peptide at the C-terminus [48]. Furthermore, in eukaryotes many of the precursor peptides possess a signal sequence on the N-terminus, before the leader peptide, which will direct the peptide to specific compartments in the cell where post-translational modification will take place [45].

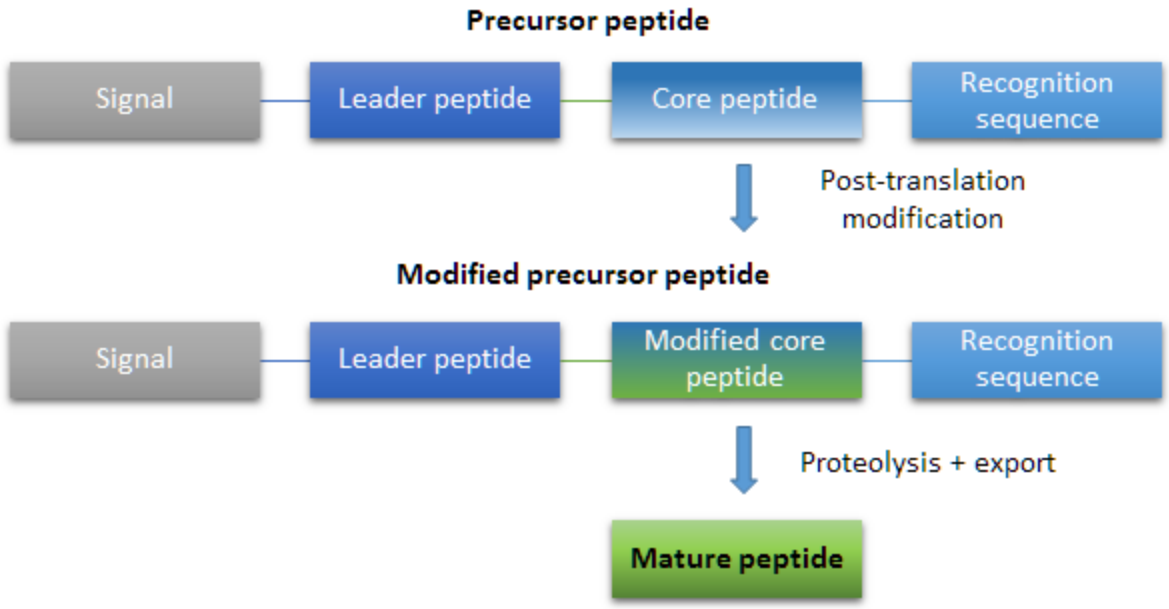


Figure 1.1 General biosynthetic pathway for ribosomally synthesised antimicrobial peptides. Adapted from Arnison *et al.* [43].

1.2.2 Modes of action

1.2.2.1 General

Antimicrobial peptides predominantly protect their host by targeting the invading microorganism using a non-selective membrane-permeablising mode of action [8,11,12,18]. This membrane-mediated mode of action is dependent on the physiochemical properties of the target cell membrane, such as charge, conformation, hydrophobicity and amphipathicity, and also environmental factors like pH and ionic strength [49,50]. In addition to the membrane-mediated mode of action, several studies have shown that antimicrobial peptides possess various intracellular targets affecting nucleic acid synthesis, protein synthesis, enzymatic activity and/or oxidative stress [9,10,51,52].

1.2.2.2 Antibacterial activity

The antibacterial mode of action is by far the most studied and well characterised mode of action of antimicrobial peptides. In the early years of antimicrobial peptide discovery it was suggested that the activity of these peptides against bacteria was because of their membrane-permeablising characteristics. However, several studies have shown that various antimicrobial peptides possess alternative modes of action or even multiple cellular targets [53-55]. Therefore, the different antibacterial modes of action will be discussed in terms of membrane-permeabilising mode of action and alternative modes of action.

Membrane-permeabilising mode of action

Regardless of their mode of action, most antimicrobial peptides have to interact with the bacterial cytoplasmic membrane. The majority of antimicrobial peptides possess three important properties that aid in their interaction with the bacterial membrane. Firstly, most of these peptides have a net positive charge that stimulates their initial attraction to the bacterial membrane [56,57]. Bacteria possess negatively charged molecules on their outer bacterial envelope, for example, lipopolysaccharides on Gram-negative bacteria and lipoteichoic acids in the case of Gram-positive bacteria [58]. Consequently, cationic antimicrobial peptides are able to bind electrostatically to these anionic components on the bacterial cell surface. Conversely, eukaryotic cell membranes are in a zwitterionic state, therefore antimicrobial peptides possess less electrostatic attraction to the cellular membranes of eukaryotic cells, providing antimicrobial peptides with some form of selectivity towards bacterial cell membranes [59]. The second and third properties enabling antimicrobial peptides to interact with bacterial membranes are amphiphaticity and structural flexibility which provides antimicrobial peptides with the ability to insert into the bacterial membranes [60].

The detailed processes that take place during cell membrane permeabilisation are not fully understood. However, several models have been developed in an attempt to explain these

processes: barrel-stave, carpet, toroidal pore and aggregate models (Figure 1.2). The barrel-stave model (Figure 1.2) explains the mode of action of antimicrobial peptides such as alamethicin and gramicidin S [61,62]. Peptides accumulate on the membrane surface until a threshold is reached resulting in clusters of peptides reorienting themselves perpendicularly to the plane of the membrane [63]. The hydrophobic regions of the peptide align with the lipid core and the hydrophilic regions face the lumen of the resulting pore [63,64]. These pores in turn make the membrane permeable towards various cytoplasmic and external ions and molecules, thus disrupting normal cell homeostasis and eventually leading to cell lysis.

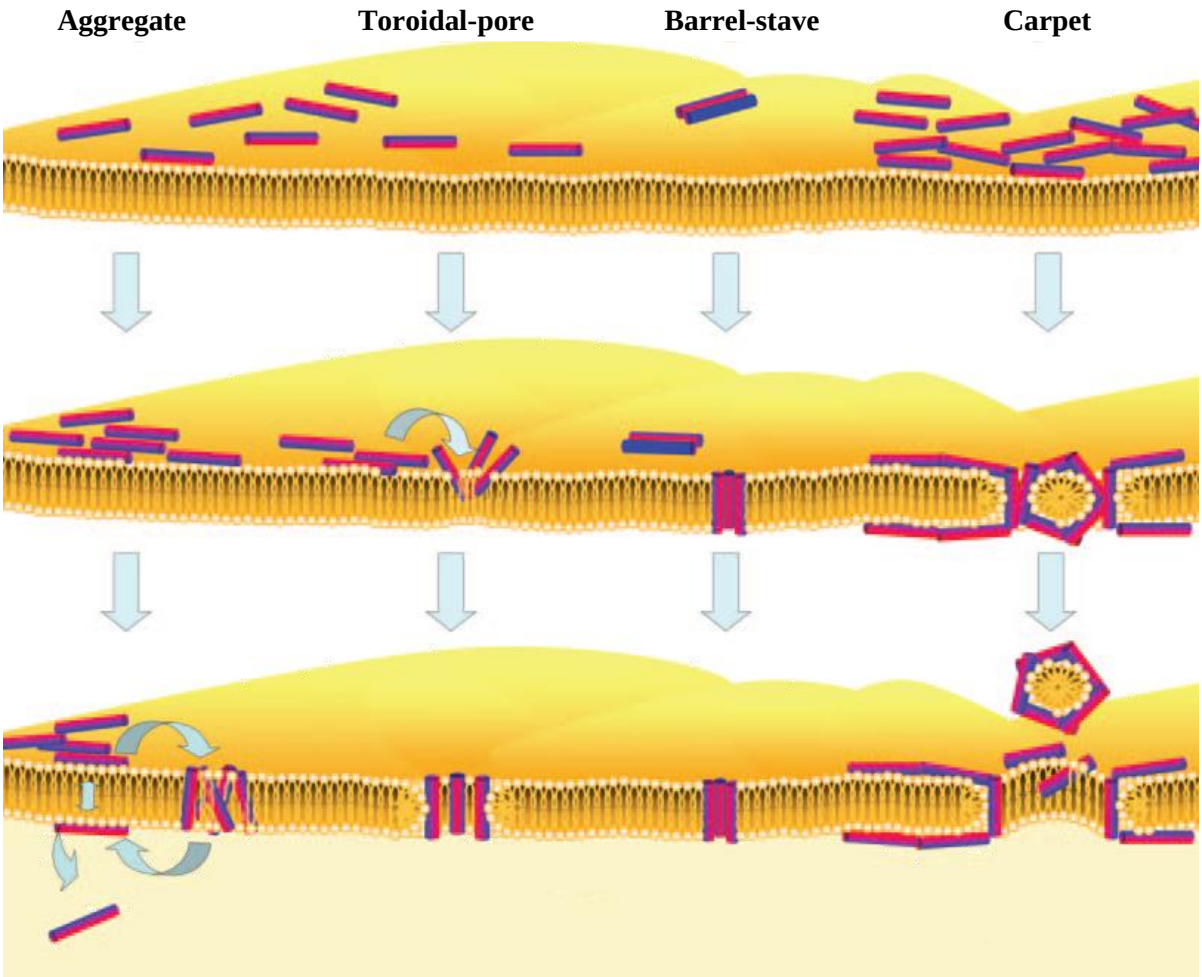


Figure 1.2 Membrane-permeabilizing mode of action models of antimicrobial peptides. Obtained from Jenssen *et al.* [52].

In the carpet model (Figure 1.2), the peptides aggregate on the bacterial cell membrane and orientate parallel to the membrane surface [65]. Once again, the cationic peptides are attached by electrostatic interactions to the anionic phospholipid head groups of the bacterial cell membrane [65], coating local areas of the membrane with a carpet-like structure. If the peptide concentration is increased above the threshold, the peptides will start to disrupt the cellular membrane in a detergent-like manner eventually leading to micelle formation which break away and forms pores in the membrane [66,67].

Most antimicrobial peptides with a cationic, amphipathic nature will cause this type of cell disruption in high enough concentrations [62]. Therefore, it is difficult to distinguish between peptides that have a true carpet mode of action from those that do not. However, mechanistic studies have showed that there are peptides, such as ovispirin, that can disrupt the bacterial cell membrane through the carpet model of membrane-mediated antibacterial activity at low peptide concentrations close to their minimal inhibition concentration (MIC) [68].

The toroidal-pore model (Figure 1.2) is similar to the barrel-stave model, apart from the phospholipid head group orientation. In this scenario, the peptide aggregates are electrostatically bound to the phospholipid head groups after which they are inserted into the cellular membrane [60,67]. Unlike the barrel-stave model, the peptides stay bound to the phospholipid head groups and the membrane lipids bend inwards to form a continuous bend [69]. The resulting toroidal pore consists of phospholipid head groups and peptides [69]. Magainins, melittin and protegrin are examples of antimicrobial peptides that have been proposed to utilise this model of membrane-mediated antibacterial activity [64,67,70].

The last model describing membrane-mediated antibacterial activity, the aggregate model (Figure 1.2), was suggested by Wu *et al.* [50]. This model, similar to the toroidal pore model, predicts that after aggregation, peptides will orientate themselves to span the membrane as aggregates with micelle-like complexes of peptides and lipids [71]. However, unlike the

toroidal pore model, peptides do not orientate in a specific orientation [50,71]. Furthermore, this model predicts that these peptides will form dramatically different channels that are able to translocate across the bacterial membrane, resulting in a negative curvature strain [71]. This model explains both membrane-permeabilising activities and translocation of peptides across bacterial membranes.

Alternative mechanisms of action

All antimicrobial peptides have to interact with the cellular membrane in some way, however not all antimicrobial peptides cause membrane permeabilisation or lysis. Several antimicrobial peptides have been discovered that cause cell death, but not membrane permeabilisation at their MIC, thus confirming the existence of intracellular modes of action [72,73]. These modes of action include inhibition of essential cellular processes like nucleic acid synthesis, cell wall synthesis, enzymatic activity and protein synthesis [10].

The antimicrobial peptide buforin II makes use of an interesting mechanism of translocation into the cytoplasm without affecting the cellular membrane integrity [74]. It is a linear α-helix peptide with a proline hinge [74]. These peptides translocate across the membrane and bind to DNA and RNA in the cytoplasm [74,75]. A study done by Park *et al.* [74] demonstrated that the proline hinge is essential for the translocation of buforin II. They showed that substitution of proline in the hinge with various other amino acids lead to a drastic decrease in antimicrobial activity, thus illustrating the essential role of the proline hinge in the antimicrobial activity of buforin II [74].

Several different classes of antimicrobial peptides such as defensins and indolicidins have been shown to exert antimicrobial activity by inhibiting nucleic acid synthesis by, as of yet, unknown mechanisms [73,76]. Additionally antimicrobial peptides like dermaseptin and pleurocidin have been shown to directly inhibit protein synthesis [72]. Various peptides that inhibit diverse cellular enzymatic activities also exist. An example of an enzymatic inhibitory

peptide is pyrrhocidin, which, after cell entry, inhibits the ATPase activity of DnaK, a protein involved in chaperone-assisted folding [77]. Pyrrhocidin prevents the correct folding of proteins which in turn leads to an accumulation of misfolded proteins and eventually cell death. Lastly, several peptides are responsible for the prevention or inhibition of cell wall synthesis. Two examples of such peptides are mersacidin and nisin, which inhibits transglycosylation or binds to lipid II, respectively [78,79]. Lipid II, an integral cell wall component, is essential in the synthesis of peptidoglycan and therefore these antimicrobial peptides prevent or inhibit cell wall formation.

It is evident that antimicrobial peptides have many diverse modes of action against bacteria. It is also possible that the modes of action may differ according to the target cell or environmental conditions. Furthermore, it has been proposed that antimicrobial peptides may combine multiple modes of actions which target essential pathways and/or structures in bacterial cells, making it extremely difficult for the development of resistance to antimicrobial peptides by pathogens [9,10,80].

1.2.2.3 Antiviral activity

Antiviral activity has been observed in a wide variety of antimicrobial peptides. These peptides are mostly active against DNA and RNA envelope viruses [11]. Furthermore, the diverse array of these peptides again have multiple modes of actions against viruses ranging from the blocking of cell to cell spread, to interactions with the viral envelope proteins [11].

Glycosaminoglycan side chains are covalently linked to proteoglycans that play important roles in eukaryotic cells and on cell surfaces [81]. The degree of sulfation of glycosaminoglycan side chains makes them among some of the most anionic compounds on the mammalian cell surface, thus facilitating the binding of cationic molecules such as virus envelope proteins [81]. The glycosaminoglycan, heparan sulfate, is one of the most important viral attachment molecules on the surface of mammalian cells [82]. The cationic nature of

antimicrobial peptides enables them to bind to heparan sulfate and thus block the binding of viruses. For example, lactoferricin molecules are able to bind to heparan sulfate which can block infection by herpes simplex virus (HSV) [83]. Lactoferricin has also demonstrated the ability to block the crossing of HSV over tight junctions, thus demonstrating another form of antiviral activity by blocking cell to cell viral spread [83]. Polyphemusin analogue T22 demonstrated another mode of action involving the host cell membrane. This antiviral peptide is able to interact with one of the co-receptors of human immunodeficiency virus type 1 (HIV-1) on T-cells, namely chemokine receptor type 4 (CXCR4) [84]. This interaction leads to a decrease in HIV-1 entry [84]. Accordingly antimicrobial peptides can also protect cells against viral entry by interaction with various host cell viral receptors.

Further modes of action of antiviral peptides are mostly directed towards the envelope and associated viral proteins. As seen in the antibacterial modes of action, antimicrobial peptides are able to interact with membranes resulting in pore formation, disruption and finally, cell lysis, thus making the viral envelope a promising target for antiviral activity [85]. It has been shown that dermaseptin has antiviral activity against HIV prior to viral entry [86]. This peptide directly interacts with viral particles leading to the disruption of the viral envelope [86]. A study done by Murakami *et al.* [87] provided direct evidence that peptides can interact and disrupt viral envelopes. Vesicular stomatitis virus (VSV) that was treated with the peptide tachyplesin-1 displayed damaged and naked virions in electron micrographs of the VSV particles, indicative of viral envelope destruction. Furthermore, interactions of antimicrobial peptides with glycoproteins on the viral envelope, as seen by the interaction of θ-defensin with the HSV glycoprotein B, which protected host cells from HSV infection, is another antiviral mode of action [88,89]. Although several modes of action of antiviral peptides have been proposed, there is still very little understood in precisely how these

mechanisms work. Consequently, further studies are required to understand the precise mode of action of these peptides.

1.2.2.4 Antifungal activity

In recent years various antimicrobial peptides have been identified that are active against fungi. Their modes of action include predominantly membrane-disruptive activity, interference with cell wall synthesis, induction of depolymerisation of actin cytoskeletons and disruption of intracellular targets [85]. Antifungal peptides can be grouped as morphogenic and non-morphogenic depending on whether or not they induce hyperbranching [90].

One of the key modes of action of antifungal peptides is proposed to be the disruption of the fungal membrane [91]. This mode of action is very similar to membrane-permeabilising modes of action in bacteria and can also be described by the various models, namely the barrel-stave, carpet, toroidal pore and aggregate models [52,85]. Furthermore, certain antimicrobial peptides have been found to influence cell wall synthesis as in the case of the plant derived peptide, *Pharbitis nil* antimicrobial peptide 1 (Pn-AMP1) [92]. It has been shown that this peptide is able to cause depolymerisation of the actin cytoskeleton of both *Saccharomyces cerevisiae* and *Candida albicans* [92].

The accumulation of reactive oxygen species (ROS) in yeast cell stimulates apoptosis [93] thus it has been proposed that certain antimicrobial peptides exert their antifungal activity via the formation of ROS [94]. One such antimicrobial peptide is the plant defensin, *Raphanus sativus* antifungal peptide 2 (RsAFP2), which has been shown to induce the formation of ROS in *C. albicans* [95].

A final mode of antifungal action is the induction of hyperbranching, although very little is understood about this mode of action. A study by Munoz *et al.* [96] on the mode of action of the hexapeptide PAF26 towards *Penicillium digitatum*, supported this mode of action. They

found that although PAF26 did not cause the membrane of the cells to become permeable, the peptide was still able to inhibit *P. digitatum* growth. They also found that treatment with PAF26 resulted in abnormal hyphal branching. Thus, it has been proposed that an antifungal peptide translocates across the fungal cell membrane into the cytoplasm where it interacts with and disturbs different regulatory machinery responsible for the normal growth and cell cycle of the fungi [96]. Although various modes of action have been proposed, the antimicrobial peptide, tyrocidine, has been found to utilise multiple modes of action including membrane disruption, ROS increase and hyperbranching towards different fungal pathogens [97,98]. Antimicrobial peptides may therefore incorporate several combinations of cellular targets and modes of action to elicit their antifungal activity.

1.2.2.5 Antiparasitic activity

Since the first discovery of antiprotozoan peptides in the 1980's, namely the magainins, several other antimicrobial peptides have been demonstrated to have antiprotozoan/antiparasitic activity [11,99]. The specific modes of action, however, are still unclear.

It has been proposed that the antiparasitic modes of action share similarities with the antibacterial, antifungal and antiviral modes of action [11]. An example is the membrane disrupting activity of defensins and cathelicidins against *Trypanosoma brucei*, which causes sleeping sickness [100]. A study done on bovine myeloid antimicrobial peptide 28 (BMAP-23) showed that this peptide exerts antiparasitic activity towards several parasites [101]. It was found that only high concentrations of BMAP-23 lead to membrane lesions, whereas low concentrations did not affect membrane integrity, however, the mitochondrial potential of the parasites was affected [101]. This indicates that antiparasitic activity might also be directed towards intracellular targets. A study by Saugar *et al.* [102] illustrated the ability of histatin 5 to permeabilise the cellular membrane of the human parasitic protozoa *Leishmania*. In

addition, the authors also demonstrated histatin 5 to elicit its antimicrobial activity by inhibiting F_1F_0-ATPase activity, resulting in the rapid decrease in mitochondrial ATP which eventually led to cell death. It has also shown that certain antimicrobial peptides such as cecropin, magainin, demaseptin, defensins, gramicidin and tyrocidine are active against the malarial parasites, *Plasmodium* spp., however, little is known about their modes of action [103-105].

1.2.3 Resistance towards antimicrobial peptides

Microorganisms have developed or obtained a diverse array of resistance strategies against antibiotics by either genetic mutations or horizontal gene transfer from the original antimicrobial producing organism. These strategies can broadly be divided into three groups: counter mechanisms of antimicrobial peptide membrane attachment and lysis, destruction or modification of the antimicrobial peptides and the transport of antimicrobial peptides away from target cites [106]. Compared to conventional antibiotics, however, antimicrobial peptide resistance mechanisms are less common and less effective than antibiotic resistance mechanisms. Steinberg *et al*. [107] demonstrated that after only 11 transfers of *Pseudomonas aeruginosa* grown in sub-MIC levels of gentamicin, resulted in a 190-fold increase in the MIC of the antibiotic. In contrast, similar conditions resulted in no significant decrease in the MIC of the antimicrobial peptide, protegrin-1 [107]. The antimicrobial peptide nisin has been used for over 50 years as food preservative without any significant increase in resistance [52]. Although less common than antibiotic resistance, resistance mechanisms towards antimicrobial peptides have been observed [106].

Staphylococcus aureus acquires antibiotic resistance by modifying its cell membrane. The *dlt* operon in *S. aureus* encodes for proteins that transport the amino acid D-alanine (D-Ala) to the cell surface [108]. Once at the cell surface D-Ala is linked to the highly anionic phosphate groups of the teichoic acid by an esterification reaction which reduces the net negative charge

of the cell membrane [108]. This protects the membrane against electrostatic interactions with cationic antimicrobial peptides. Furthermore, *S. aureus* also possesses a lysylphosphatidylglycerol (LPG) synthetase, encoded by the multiple peptide resistance factor (mprF) gene, which is responsible for the production of LPG (D-lysine bound to a phosphatidylglycerol) [109]. The LPG molecule is able to insert into the cell membrane which also increases the positive charge [109]. Gram-negative bacteria are able to induce resistance by altering the charges on the lipid A moiety of their lipopolysaccharide [110,111]. The Gram-negative bacteria, *Yersinia enterocolitica*, incorporates the protein YadA (*Yersinia* adhesion A domain), encoded by the virulence plasmid pYVe, into its outer membrane [112]. This membrane alteration assists the bacterium in resistance towards killing by antimicrobial polypeptides produced by human granulocytes [112].

Bacteria also utilise proteolytic enzymes as resistance mechanisms. These are prominent mechanisms of resistance against conventional antibiotics [106]. However, the involvement of this mechanism in resistance towards antimicrobial peptides is still controversial. One example is the inactivation of the antimicrobial peptide, LL-37, by the *S. aureus* metalloproteinase, aureolysin [113].

The transport of unwanted peptides across the membrane is another method of antimicrobial resistance. One of the predominant transport-mediated resistance mechanisms is through the multidrug-resistance (MDR) efflux pump [114]. This mechanism is essential for the resistance of microorganisms to antimicrobial peptides and antibiotics that have intracellular targets. An example where this mechanism is implemented is in *Meningococci* where the MDR-pump is able to confer resistance towards LL-37, polymyxin B and protegrine-1 [115]. *Yersinia* confers its resistance towards the antimicrobial peptide polymyxin B via an efflux pump/K^+ antiport system which pumps polymyxin B back out of the cell to prevent toxicity [116]. It has also been suggested that antimicrobial resistance can be achieved by the uptake

of peptides into the cell via the ATP-binding cassette transporter followed by proteolytic breakdown in the cytoplasm [117].

A final method of resistance found in bacteria is biofilm formation, where the biofilm consists of a thick microbial aggregate of slow growing cells [118]. The biofilm limits antimicrobial penetration and although the top cell layers are exposed to the antimicrobial agent, the cells in the bottom layers are sheltered and therefore protected [118].

1.2.4 Practical applications of antimicrobial peptides

Antimicrobial peptides have become convincing therapeutic candidates in the clinical and agricultural sectors due to their diverse and fast acting modes of action, low potential for resistance development and the resistance of pathogens towards current, conventional antibiotics. A vast majority have served as lead compounds for the development of clinical antibiotics, however, few have been approved [1]. Several peptides have been used clinically for many years as topical antibiotics [119-121]. Daptomycin, one of the most bactericidal drugs, is used as reserve antibiotic to treat opportunistic Gram-positive infections [122]. One of the most well studied antimicrobial peptides, nisin from the bacterium *Lactococcus lactis*, has been used for years as a food preservative [123]. This demonstrates the important role that these molecules already have in practical applications.

Antimicrobial peptides have been considered for systemic application in the clinical setting. However, due to cost, poor pharmacokinetic data and unknown toxicity of antimicrobial peptides they have mostly been limited to topical uses [1]. The treatment of acne, caused by *Propionibacterium acnes*, with antimicrobial peptides seems to have a promising future. The indolicin-derived peptide, MBI-594AN, is active against *P. acnes* and has anti-inflammatory activity by the suppression of *P. acnes* stimulated cytokine release [124]. Furthermore, studies are currently underway to determine if bioactive coatings with MBI-594AN on

surgical implants, needles, catheters etc. will protect against pathogen colonization and infections [125]. Several antimicrobial peptides also possess immunomodulatory activities suggesting they may have potential applications in wound healing, vaccine adjuvants and anticancer drugs [1].

In recent years the agricultural sector has focused on producing eco-friendly and natural or organic crops by employing various microorganisms into soil additives to control plant pathogens as an alternative to conventional pesticides [126-128]. Nowadays, several products that incorporate microorganisms to produce antimicrobial peptides as pathogen controlling agents are available, such as MYCOSTOP® and Kodiak [129]. However, this is not always an efficient method of controlling plant pathogens as some antimicrobial peptides may not be taken up by the plant or may be synthesised only after the plant is infected with harmful levels of pathogens. These problems could be overcome by genetically manipulating plants to endogenously express certain antimicrobial peptides, thereby ensuring that the peptides are present in the plant tissue prior to infection [130]. However, modern day consumers are cautious towards genetically modified foods and prefer natural or organic products. By using naturally-derived antimicrobial peptides, both the above mentioned problems are circumvented.

Several characteristics of antimicrobial peptides make them desirable for use as antibiotics in both the clinical and agricultural sectors, however, their application does have limitations. A primary disadvantage of some antimicrobial peptides in clinical applications is their toxicity. For example, the cyclic antimicrobial peptide, gramicidin S, is effective against a broad spectrum of bacteria and even parasites [104,131], however, this peptide is highly haemolytic and its use is therefore restricted to topical applications to address surface infections [132]. A potential solution to this toxicity is the modification of gramicidin S by substitution of

various amino acids to optimally increase its therapeutic index (selectivity towards pathogen cells as opposed to host cells) [133].

A further limitation is that antimicrobial peptides are subject to protein degradation by proteases which leads to undesirable pharmacokinetics. Several different methods exist to overcome this limitation, such as chemical modifications including: the incorporation of unusual or D-amino acids, cyclisation, formulations to improve stability, the development of the peptide as a prodrug or the use of non-peptide backbones [134].

Possibly the biggest limitation of antimicrobial peptides is the cost of large scale production. The production of antimicrobial peptides is usually more expensive than conventional antibiotics as peptide synthesis is expensive and often require multiple purification steps. The disadvantage of expensive chemical synthesis for ribosomally synthesised peptides can for larger peptides be overcome by the use of recombinant DNA technology. However, limitations may arise as these peptides are antimicrobial in nature and therefore may kill the host producing organism. Furthermore, various antimicrobial peptides are non-ribosomally synthesised by large multienzyme molecules with complex genetics, thus excluding these peptides for the use in recombinant DNA technology [13]. However, research into the development of synthetic peptides, peptidomimics and peptoids (N-alkylglycine polymers) is rapidly advancing and has the potential to overcome most of the above mentioned limitations [135,136].

1.3 Soil and environmental bacteria: an invaluable source of novel antimicrobial peptides

With the ‘de-evolution’ back to a time were the use of antibiotics to treat infectious disease is becoming an increasing impossibility due to antibiotic resistance, emphasis is being placed on novel antibiotic discovery. Furthermore, focus is shifting from classical antibiotics to new

classes of antimicrobial agents such as antimicrobial peptides. However, with these peptides being produced by species from all kingdoms of life, the decision on where to start the search can be a daunting one at best. Nevertheless, soil bacteria have historically been implicated in producing the largest variety of clinically viable antibiotics and have therefore once again became the central focus of novel antimicrobial peptide discovery [137].

Soil largely consists of solid-phase soil particles which provide microorganisms with adhesion sites and pores where microbial micro-colonies can be established [138]. Furthermore, the soil environments are predominantly static and devoid of substantial mixing. The structural matrix and minimal mixing leads to the formation of microhabitats within the soil [139]. Thus, microorganism metabolism and interactions are limited to conditions at a microhabitat level, which in turn results in a large variety of microbial niches [140]. The immense diversity in soil bacteria and the deficiency of nutrients due to the static environment, subsequently results in inter-species competition for nutritional resources [141-143]. Competition results in further diversification of bacteria to evolve various mechanisms to outcompete rivals [141]. Examples of strategies to outcompete surrounding microorganisms include acid production, increased growth rates, biofilm formation and production of antimicrobial compounds [143-148]. The resulting microbial heterogeneity and competition explains why the bacterial diversity in the soil environment surpasses that of any other environment [149].

The discovery of penicillin in 1929 by Alexander Fleming [150] and its subsequent clinical use in 1945 due to extraction techniques developed by Chain *et al* [151], seized the attention of many researchers. This lead to a “golden era” of antibiotic discovery during which most of the antibiotics used today were discovered [137]. During the same era, Rene Dubos discovered the first antimicrobial peptide complex, tyrothricin, from the soil bacterium *Bacillus brevis* (now called *Brevibacillus parabrevis*) [21]. In 1940 Dubos and Hotchkiss

[152] showed that the tyrothricin complex comprised of two antimicrobial polypeptides, which was subsequently named gramicidin and tyrocidine [153]. The therapeutic potentials of gramicidin and tyrocidine were subsequently studied in 1944 resulting in their application as the first topical antibiotics, which are still being used today [120,154]. However, it was only after the discovery of the first aminoglycoside antibiotic, streptomycin produced by the soil bacteria *Streptomyces griseus*, that a worldwide scavenge for antibiotics from soil bacteria originated [137,155]. Hundreds of antibiotics were subsequently discovered with varying chemical structures and antimicrobial activities [137]. This finally led to a decreased interest in antimicrobial peptides as β-lactam-, tetracycline-, aminoglycoside- and macrolide antibiotics were shown to generally have better antibacterial activity and lower cytotoxicity [156]. However, with the increase of antimicrobial resistance towards conventional antibiotics, the possibility of antimicrobial peptides being used as antimicrobial chemotherapy has been resurrected.

Antimicrobial peptides from soil bacteria are classified into two broad groups according to their biosynthesis. The first group of antimicrobial peptides, bacteriocins, are ribosomally produced by bacteria. The bacteriocins from *Escherichia coli* strains have been the focus of most of the research done on antimicrobial peptides from Gram-negative bacteria due to their routine use in laboratories [157,158]. Consequently, bacteriocins from Gram-negative bacteria are categorized into two groups, colicins and microcins [157-161]. Colicins are large peptides produced by *E. coli* under stressful conditions with activity to closely related bacterial species [162]. They exert their antimicrobial activity by either voltage-dependent pore formation in the cell membrane, peptidoglycan destruction or by a nuclease activity in the cytoplasmic environment [163-167]. Microcins, on the other hand, are small peptides that are generally heat-stable, stable in high pH and resistant to protease degradation [157,160,161]. Like colicins, microcins possess various modes of action both membrane-

mediated and intracellularly against phylogenetically related organisms [168-171]. *E. coli* and other members of the family *Enterobacteriaceae*, that have been responsible for most of the bacteriocins from Gram-negative bacteria studied to date, are predominantly found in the mammalian intestinal tract [172]. However, many of these species have also been isolated from soil and, therefore, can be included into the antimicrobial peptide library from soil environments [173].

Bacteriocins from Gram-positive bacteria consist of peptides with a variety of structures. They possess activity predominantly towards bacterial strains that are closely related to the producer strain with some exceptions [174]. Bacteriocins, from Gram-positive bacteria, were first classified by Klaenhammer [175] into four classes based on chemical structure, mode of action and occurrence of modified amino acids. Class I bacteriocins, also referred to as lantibiotics, are small peptides that were modified by post-translational modification to contain the modified amino acids lanthionine (thioether linkage of two alanine residues) and often β-methyl lanthionine [176,177]. These peptides exert their antimicrobial activity through various modes of action including: membrane pore formation, cell wall synthesis disruption and inhibition of spore growth [178-180]. An antimicrobial peptide produced by the lactic acid bacterium *Lactococcus lactis* first described in 1928 and subsequently named nisin in 1947, became one of the most well-known and characterised peptides from this class [181-183]. It is still routinely used as a biopreservative to prolong the shelf life of various foods [183]. The second class of bacteriocins (class II) are small heat-stable peptides that do not contain unusual amino acids and exert their antimicrobial activity mostly via cell membrane disruption [174,184,185]. This group is further subdivided into class IIa (pediocin-like peptides with activity against *Listeria*), class IIb (multi-peptide bacteriocins) and class IIc bacteriocins (miscellaneous unmodified bacteriocins) [174,184,185]. Class III bacteriocins (bacteriolysins) are larger (> 10 kDa) peptides that are heat labile [174,184]. The

name bacteriolysin refers to the ability of these peptides to lyse the cellular membrane of target bacterial cells [184]. The last group of bacteriocins (class IV) has not been as extensively studied as the other classes. This group contains complex proteins that often have lipid or carbohydrate moieties incorporated into their structure [42]. Bacteriocins of the bacterial clad lactic acid bacteria (LAB) have been the subject of the majority of research done on bacteriocins due to their generally recognised as safe (GRAS) status and use in the food industry [186]. Although they are not strictly soil bacteria, various species of LAB have been isolated and characterised from soil environments [187]. Bacterial species from the genus *Bacillus*, which are predominantly found in soil, have also been shown to produce a large variety of bacteriocins that can be categorized into the first three classes of the bacteriocins from Gram-positive bacteria [188]. Most bacteriocins produced by *Bacillus* spp. were found to have a broader spectrum of antimicrobial activity compared to LAB, with some indicating activity against methicillin resistant *S aureus* (MRSA) [188-190]. Examples of *Bacillus* produced bacteriocins include: bacillin, subtilosin A, amylolysin, coagulin and megacin [191-195].

The second broadly classified group of antimicrobial peptides from soil bacteria is non-ribosomally synthesised peptides, sometimes referred to as peptide antibiotics. Several structural features as mentioned earlier clearly distinguish these peptides from bacteriocins and provide them with large structural diversity [13]. Several non-ribosomally produced peptides from soil organisms have an important role in the pharmaceutical sector today. A Glycopeptide antibiotic, vancomycin, isolated from the soil bacterium *Streptomyces orientalis* is one of the most important ‘last resort’ antibiotics used today to treat various infections [196]. The cytotoxic activity of the non-ribosomal peptide bleomycin, produced by the soil bacterium *Streptomyces verticillus*, resulted in its use as an anticancer medication for various cancers including: testicular cancer, ovarian cancer, Hodgkin’s lymphoma, non-

Hodgkin's lymphoma etc. [197]. Furthermore, several *Bacillus* strains produce biosurfactants that possess antimicrobial activity such as the iturin and surfactin group of lipopeptides [198,199]. These peptides are predominantly used in pollution remediation, however, recently they have been investigated for their potential use as antimicrobial agents in the pharmaceutical sector [198].

Br. parabrevis produces at least 28 peptide analogues of the antimicrobial peptide group collectively referred to as tyrocidines [200]. These peptides are non-ribosomally synthesised together with linear gramicidins to form a secondary metabolite complex named tyrothricin [200,201]. The tyrocidines can be seen as cyclic decapeptide analogues of gramicidin S as they possess a shared pentapeptide unit namely L-Val^{8}-L-Orn^{9}-L-Leu^{10}-D-Phe^{1}-L-Pro^{2} that can vary in position 8 and 9 [202,203]. The second peptide unit of tyrocidines, however, can vary in positions 3, 4 and 7 an can be defined as L-Tyr/Phe/Trp^{3}-D-Tyr/Phe/Trp^{4}-L-Asn^{5}-L-Gln^{6}-L-Tyr/Phe/Trp^{7} [200]. Tryptocidine C, with a Trp in positions 3, 4 and 7 is one of the antimicrobial peptides focused on in this study. Antimicrobial activity of tyrocidins, including tryptocidine C, has been observed for various Gram-positive and Gram-negative bacteria, fungi and the malarial parasite *Plasmodium falciparum* [21,98,104,204,205]. The mode of action of these peptides has not been fully characterised, but several studies suggest a membranolytic mode of action [205-207]. However, Mach and Slayman [208] suggested intracellular mode of actions including the inhibition of DNA and RNA synthesis.

Bacitracin, another important antimicrobial peptide included in this study, was first discovered by Johnson *el al*. [209] in 1945 from a strain of *Bacillus licheniforms* isolated from a wound on a young girl named Margaret Tracy. Bacitracin has been shown to have potent activity mostly towards Gram-positive bacteria including *Staphylococcus* spp. and *Streptococcus* spp. [209,210]. Today it is used as a topical antibiotic to treat localized skin as well as eye infections [121]. Bacitracin could also be used as an intramuscular treatment of

staphylococcal infections, however, is avoided due to its toxicity. Commercial bacitracin is a mixture of closely related dodecapeptides with bacitracin A being the most abundant and active [211]. Bacitracin A comprises of a linear branch (L-Ile$_1$-L-thiazoline$_2$-L-Leu$_3$-D-Glu$_4$-L-Ile$_5$) which is connected to the L-lysine of a cyclic heptapeptide (L-Lys$_6$-D-Orn$_7$-L-Ile$_8$-D-Phe$_9$-L-His$_{10}$-D-Asp$_{11}$-L-Asn$_{12}$) [212]. As with most non-ribosomally synthesised peptides, bacitracin contains unusual features. The most distinctive of these features is a thiozoline ring between L-cysteine and the *N*-terminal L-isoleucine in the branched chain [213]. The primary mode of action of bacitracin has been extensively studied and shown to be due to its ability to disrupt the translocation of *N*-acetylmuramylpentapeptide across the cell membrane, which is an essential part of peptidoglycan synthesis [53]. Before translocation UDP-*N*-acetylmuramylpentapeptide is bound to C_{55}-isoprenyl phosphate by a pyrophosphate bond [214]. Bound *N*-acetylmuramylpentapeptide is then translocated across the cellular membrane followed by its incorporation into peptidoglycan [214]. C_{55}-isoprenyl pyrophosphate is then translocated back to the cytoplasm where it is dephosphorylated back to C_{55}-isoprenyl phosphate by a membrane associated pyrophosphatase [215]. Bacitracin has been shown to form a divalent metal ion dependent complex with C_{55}-isoprenyl pyrophosphate, resulting in the inhibition of dephosphorylation [53]. UDP-*N*-acetylmuramylpentapeptide can only react with the monophosphate form of C_{55}-isoprenyl [53]. Bacitracin, therefore, ultimately results in the disruption of *N*-acetylmuramylpentapeptide translocation and inhibits the subsequent peptidoglycan formation. In the absence of peptidoglycan's restraining influence, the cellular membrane will ultimately be lysed due to the high intracellular osmotic potential. In addition to disrupting peptidoglycan synthesis, bacitracin has also been shown to lyse protoplasts prepared from *B. licheniforms* and *Micrococcus lysodeikticus* (now called *Micrococcus luteus)* at concentrations similar to its MIC [216]. However, whether this membranolytic

interaction is a prominent mode of action or only aids in the inhibition of peptidoglycan synthesis is still subject to further studies.

Historically antimicrobial peptides from soil bacteria appear to be intensely studied and drained of novelty. However, only a small percentage of the earth's soil surface has been studied and screened for antimicrobial agents [217], therefore providing enormous potential for the discovery of novel antimicrobial peptides. Furthermore, it has been shown through genomic studies that only 1% of the microorganisms found in soil can be cultured with current traditional laboratory techniques [218]. These "uncultivable" microorganisms, referred to as "microbial dark matter", might hold the key for opening the door to a variety of novel antimicrobial agents [140,219]. Several attempts have been made to culture microorganism from the "microbial dark matter" of the soil biome [140]. One recent success in the development of new culture techniques has led to the discovery of the novel antimicrobial peptide, teixobactin. Ling *et al*. [220] extracted teixobactin from a newly discovered Gram-negative β-proteobacterium named *Eleftheria terrae*. *E. terrae* was isolated from soil by an *in situ* culturing method developed by Nichols *et al*. [221] where single bacterial cells are grown in their natural soil environment separated by a semi-permeable membrane [220]. Therefore, the bacteria are allowed to grow in their natural habitat resulting in enrichment conditions that are not usually replicated in the laboratory [221]. Teixobactin was subsequently shown to be active against a large variety of Gram-positive and Gram-negative pathogenic microorganisms including MRSA [220].

The seemingly endless diversity of soil bacterial strains, as well as the structural and antimicrobial diversity of the antimicrobial agents they produce designates the soil biome as a valuable resource for the discovery of novel antimicrobial agents and more specifically antimicrobial peptides. Moreover, the small percentage of soil surface area screened and the untapped potential of the "microbial dark matter" provides researchers with the opportunity

and obligation to focus studies on novel natural antimicrobial agents from these environments, to fight against the ever increasing dilemma of antimicrobial resistance. The main goal of this project was therefore to discover novel antimicrobial peptides produced by soil bacteria so as to enlarge the antibiotic library and ultimately aid in the ever increasing crisis of resistance development by pathogens towards current antibiotics.

1.4 References

1. Hancock, R. E. W. and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. **24**, 1551-1557
2. Mor, A. (2000) Peptide-based antibiotics: a potential answer to raging antimicrobial resistance. Drug Dev. Res. **50**, 440-447
3. Hancock, R. E. and Lehrer, R. (1998) Cationic peptides: a new source of antibiotics. Trends Biotechnol. **16**, 82-88
4. Hancock, R. and Patrzykat, A. (2002) Clinical development of cationic antimicrobial peptides: from natural to novel antibiotics. Current drug targets - infectious disorders. **2**, 79-83
5. Giuliani, A., Pirri, G. and Nicoletto, S. (2007) Antimicrobial peptides: an overview of a promising class of therapeutics. Cen. Eur. J. Biol. **2**, 1-33
6. Andreu, D. and Rivas, L. (1998) Animal antimicrobial peptides: an overview. Biopolymers. **47**, 415-433
7. Epand, R. M. and Vogel, H. J. (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta. **1462**, 11-28
8. Zhang, L. and Falla, T. J. (2006) Antimicrobial peptides: therapeutic potential. Expert Opin. Pharmacother. **7**, 653-663
9. Nguyen, L. T., Haney, E. F. and Vogel, H. J. (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. **29**, 464-472
10. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. Nat. Rev. Microbiol. **3**, 238-250
11. Brandenburg, L., Merres, J., Albrecht, L., Varoga, D. and Pufe, T. (2012) Antimicrobial peptides: multifunctional drugs for different applications. Polymers. **4**, 539-560
12. Sang, Y. and Blecha, F. (2008) Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Anim. Health Res. Rev. **9**, 227-235
13. Schwarzer, D., Finking, R. and Marahiel, M. A. (2003) Nonribosomal peptides: from genes to products. Nat. Prod. Rep. **20**, 275-287
14. Bulet, P. and Stocklin, R. (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept. Lett. **12**, 3-11

15. Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W. and Goździcka-Józefiak, A. (2014) Plant antimicrobial peptides. Folia Microbiol. **59**, 181-196

16. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature. **415**, 389-395

17. Hancock, R. E. W. (2001) Cationic peptides: effectors in innate immunity and novel antimicrobials. Lancet Infect. Dis. **1**, 156-164

18. Bradshaw, J. P. (2003) Cationic antimicrobial peptides: issues for potential clinical use. BioDrugs. **17**, 233-240

19. Koczulla, A. R. and Bals, R. (2003) Antimicrobial peptides: current status and therapeutic potential. Drugs. **63**, 389-406

20. Hoskin, D. W. and Ramamoorthy, A. (2008) Studies on anticancer activities of antimicrobial peptides. Biochim. Biophys. Acta. **1778**, 357-375

21. Dubos, R. J. (1939) Studies on a bactericidal agent extracted from a soil *Bacillus*: I. preparation of the agent. its activity *in vitro*. J. Exp. Med. **70**, 1-10

22. Wang, G., Li, X. and Wang, Z. (2016) APD3: the antimicrobial peptide database as a tool for research and education. Nucleic Acids Res. **44**, D1087-D1093

23. Marahiel, M. A., Stachelhaus, T. and Mootz, H. D. (1997) Modular peptide synthetases involved in nonribosomal peptide synthesis. Chem. Rev. **97**, 2651-2674

24. Byford, M. F., Baldwin, J. E., Shiau, C. and Schofield, C. J. (1997) The mechanism of ACV synthetase. Chem. Rev. **97**, 2631-2650

25. Vosloo, J. A., Stander, M. A., Leussa, A. N., Spathelf, B. M. and Rautenbach, M. (2013) Manipulation of the tyrothricin production profile of *Bacillus aneurinolyticus*. Microbiology. **159**, 2200-2211

26. Weber, G., Schörgendorfer, K., Schneider-Scherzer, E. and Leitner, E. (1994) The peptide synthetase catalyzing cyclosporine production in *Tolypocladium niveum* is encoded by a giant 45.8-kilobase open reading frame. Curr. Genet. **26**, 120-125

27. Du, L., Sánchez, C., Chen, M., Edwards, D. J. and Shen, B. (2000) The biosynthetic gene cluster for the antitumor drug bleomycin from *Streptomyces verticillus* ATCC15003 supporting functional interactions between nonribosomal peptide synthetases and a polyketide synthase. Chem. Biol. **7**, 623-642

28. Gehring, A. M., DeMoll, E., Fetherston, J. D., Mori, I., Mayhew, G. F., Blattner, F. R., Walsh, C. T. and Perry, R. D. (1998) Iron acquisition in plague: modular logic in enzymatic biogenesis of yersiniabactin by *Yersinia pestis*. Chem. Biol. **5**, 573-586

29. Stein, T. (2005) *Bacillus subtilis* antibiotics: structures, syntheses and specific functions. Mol. Microbiol. **56**, 845-857

30. Stein, T., Vater, J., Kruft, V., Otto, A., Wittmann-Liebold, B., Franke, P., Panico, M., McDowell, R. and Morris, H. R. (1996) The multiple carrier model of nonribosomal peptide biosynthesis at modular multienzymatic templates. J. Biol. Chem. **271**, 15428-15435

31. Stachelhaus, T. and Marahiel, M. A. (1995) Modular structure of genes encoding multifunctional peptide synthetases required for non-ribosomal peptide synthesis. FEMS Microbiol. Lett. **125**, 3-14

32. Dieckmann, R., Lee, Y. O., van Liempt, H., von Döhren, H. and Kleinkauf, H. (1995) Expression of an active adenylate-forming domain of peptide synthetases corresponding to acyl-CoA-synthetases. FEBS Lett. **357**, 212-216

33. Stachelhaus, T., Mootz, H. D. and Marahiel, M. A. (1999) The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. Chem. Biol. **6**, 493-505

34. Eppelmann, K., Stachelhaus, T. and Marahiel, M. A. (2002) Exploitation of the selectivity-conferring code of nonribosomal peptide synthetases for the rational design of novel peptide antibiotics. Biochemistry. **41**, 9718-9726

35. Stachelhaus, T., Hüser, A. and Marahiel, M. A. (1996) Biochemical characterization of peptidyl carrier protein (PCP), the thiolation domain of multifunctional peptide synthetases. Chem. Biol. **3**, 913-921

36. Walsh, C. T., Gehring, A. M., Weinreb, P. H., Quadri, L. E. and Flugel, R. S. (1997) Post-translational modification of polyketide and nonribosomal peptide synthases. Curr. Opin. Chem. Biol. **1**, 309-315

37. Keating, T. A. and Walsh, C. T. (1999) Initiation, elongation, and termination strategies in polyketide and polypeptide antibiotic biosynthesis. Curr. Opin. Chem. Biol. **3**, 598-606

38. Schwarzer, D., Mootz, H. D., Linne, U. and Marahiel, M. A. (2002) Regeneration of misprimed nonribosomal peptide synthetases by type II thioesterases. Proc. Natl. Acad. Sci. U. S. A. **99**, 14083-14088

39. Stachelhaus, T., Mootz, H. D., Bergendahl, V. and Marahiel, M. A. (1998) Peptide bond formation in nonribosomal peptide biosynthesis: catalytic role of the condensation domain. J. Biol. Chem. **273**, 22773-22781

40. Linne, U. and Marahiel, M. A. (2000) Control of directionality in nonribosomal peptide synthesis: role of the condensation domain in preventing misinitiation and timing of epimerization. Biochemistry. **39**, 10439-10447

41. Schneider, A. and Marahiel, M. A. (1998) Genetic evidence for a role of thioesterase domains, integrated in or associated with peptide synthetases, in non-ribosomal peptide biosynthesis in *Bacillus subtilis*. Arch. Microbiol. **169**, 404-410

42. Papagianni, M. (2003) Ribosomally synthesized peptides with antimicrobial properties: biosynthesis, structure, function, and applications. Biotechnol. Adv. **21**, 465-499

43. Arnison, P. G., Bibb, M. J., Bierbaum, G., Bowers, A. A., Bugni, T. S., Bulaj, G., Camarero, J. A., Campopiano, D. J., Challis, G. L., Clardy, J., Cotter, P., D, Craik, D. J., Dawson, M., Dittmann, E., Donadio, S., Dorrestein, P. C., Entian, K., Fischbach, M. A., Garavelli, J. S., Goransson, U., Gruber, C. W., Haft, D. H., Hemscheidt, T. K., Hertweck, C., Hill, C., Horswill, A. R., Jaspars, M., Kelly, W. L., Klinman, J. P., Kuipers, O. P., Link, A. J., Liu, W., Marahiel, M. A., Mitchell, D. A., Moll, G. N., Moore, B. S., Muller, R., Nair, S. K., Nes, I. F., Norris, G. E., Olivera, B. M., Onaka, H., Patchett, M. L., Piel, J., Reaney, M. J. T., Rebuffat, S., Ross, R. P., Sahl, H., Schmidt, E. W., Selsted, M. E., Severinov, K., Shen, B., Sivonen, K., Smith, L., Stein, T., Sussmuth, R. D., Tagg, J. R., Tang, G., Truman, A. W., Vederas, J. C., Walsh, C. T., Walton, J. D., Wenzel, S. C., Willey, J. M. and van der Donk, Wilfred A. (2013) Ribosomally synthesized and post-translationally modified peptide natural products:

overview and recommendations for a universal nomenclature. Nat. Prod. Rep. **30**, 108-160

44. Hancock, R. E. W. (1997) Peptide antibiotics. Lancet. **349**, 418-422
45. McIntosh, J. A., Donia, M. S. and Schmidt, E. W. (2009) Ribosomal peptide natural products: bridging the ribosomal and nonribosomal worlds. Nat. Prod. Rep. **26**, 537-559
46. Li, B., Sher, D., Kelly, L., Shi, Y., Huang, K., Knerr, P. J., Joewono, I., Rusch, D., Chisholm, S. W. and van der Donk, W. A. (2010) Catalytic promiscuity in the biosynthesis of cyclic peptide secondary metabolites in planktonic marine cyanobacteria. Proc. Natl. Acad. Sci. U. S. A. **107**, 10430-10435
47. Oman, T. J. and van der Donk, Wilfred A. (2010) Follow the leader: the use of leader peptides to guide natural product biosynthesis. Nat. Chem. Biol. **6**, 9-18
48. Huo, L., Rachid, S., Stadler, M., Wenzel, S. C. and Müller, R. (2012) Synthetic biotechnology to study and engineer ribosomal bottromycin biosynthesis. Chem. Biol. **19**, 1278-1287
49. Hancock, R. E. W. and Rozek, A. (2002) Role of membranes in the activities of antimicrobial cationic peptides. FEMS Microbiol. Lett. **206**, 143-149
50. Wu, M., Maier, E., Benz, R. and Hancock, R. E. W. (1999) Mechanism of interaction of different classes of cationic antimicrobial peptides with planar bilayers and with the cytoplasmic membrane of *Escherichia coli*. Biochemistry. **38**, 7235-7242
51. Nicolas, P. (2009) Multifunctional host defense peptides: intracellular-targeting antimicrobial peptides. FEBS J. **276**, 6483-6496
52. Jenssen, H., Hamill, P. and Hancock, R. E. W. (2006) Peptide antimicrobial agents. Clin. Microbiol. Rev. **19**, 491-511
53. Stone, K. J. and Strominger, J. L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. U. S. A. **68**, 3223-3227
54. Parks, W. M., Bottrill, A. R., Pierrat, O. A., Durrant, M. C. and Maxwell, A. (2007) The action of the bacterial toxin, microcin B17, on DNA gyrase. Biochimie. **89**, 500-507
55. Metlitskaya, A., Kazakov, T., Kommer, A., Pavlova, O., Praetorius-Ibba, M., Ibba, M., Krasheninnikov, I., Kolb, V., Khmel, I. and Severinov, K. (2006) Aspartyl-tRNA synthetase is the target of peptide nucleotide antibiotic microcin C. J. Biol. Chem. **281**, 18033-18042
56. Dathe, M., Schümann, M., Wieprecht, T., Winkler, A., Beyermann, M., Krause, E., Matsuzaki, K., Murase, O. and Bienert, M. (1996) Peptide helicity and membrane surface charge modulate the balance of electrostatic and hydrophobic interactions with lipid bilayers and biological membranes. Biochemistry. **35**, 12612-12622
57. Frecer, V., Ho, B. and Ding, J. L. (2004) De novo design of potent antimicrobial peptides. Antimicrob. Agents Chemother. **48**, 3349-3357
58. Hancock, R. E. W. (1997) The bacterial outer membrane as a drug barrier. Trends Microbiol. **5**, 37-42

59. Gumila, C., Ancelin, M. L., Jeminet, G., Delort, A. M., Miquel, G. and Vial, H. J. (1996) Differential in vitro activities of ionophore compounds against *Plasmodium falciparum* and mammalian cells. Antimicrob. Agents Chemother. **40**, 602-608

60. Mihajlovic, M. and Lazaridis, T. (2010) Antimicrobial peptides in toroidal and cylindrical pores. Biochim. Biophys. Acta. **1798**, 1485-1493

61. Spaar, A., Münster, C. and Salditt, T. (2004) Conformation of peptides in lipid membranes studied by x-ray grazing incidence scattering. Biophys. J. **87**, 396-407

62. Zhang, L., Rozek, A. and Hancock, R. E. W. (2001) Interaction of cationic antimicrobial peptides with model membranes. J. Biol. Chem. **276**, 35714-35722

63. Ehrenstein, G. and Lecar, H. (1977) Electrically gated ionic channels in lipid bilayers. Q. Rev. Biophys. **10**, 1-34

64. Yang, L., Harroun, T. A., Weiss, T. M., Ding, L. and Huang, H. W. (2001) Barrel-stave model or toroidal model? A case study on melittin pores. Biophys. J. **81**, 1475-1485

65. Pouny, Y., Rapaport, D., Mor, A., Nicolas, P. and Shai, Y. (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. Biochemistry. **31**, 12416-12423

66. Ladokhin, A. S. and White, S. H. (2001) 'Detergent-like'permeabilization of anionic lipid vesicles by melittin. Biochim. Biophys. Acta. **1514**, 253-260

67. Matsuzaki, K., Murase, O., Fujii, N. and Miyajima, K. (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry. **35**, 11361-11368

68. Yamaguchi, S., Huster, D., Waring, A., Lehrer, R. I., Kearney, W., Tack, B. F. and Hong, M. (2001) Orientation and dynamics of an antimicrobial peptide in the lipid bilayer by solid-state NMR spectroscopy. Biophys. J. **81**, 2203-2214

69. Ludtke, S. J., He, K., Heller, W. T., Harroun, T. A., Yang, L. and Huang, H. W. (1996) Membrane pores induced by magainin. Biochemistry. **35**, 13723-13728

70. Yamaguchi, S., Hong, T., Waring, A., Lehrer, R. I. and Hong, M. (2002) Solid-state NMR investigations of peptide-lipid interaction and orientation of a β-sheet antimicrobial peptide, protegrin. Biochemistry. **41**, 9852-9862

71. Bechinger, B. and Lohner, K. (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim. Biophys. Acta. **1758**, 1529-1539

72. Patrzykat, A., Friedrich, C. L., Zhang, L., Mendoza, V. and Hancock, R. E. W. (2002) Sublethal concentrations of pleurocidin-derived antimicrobial peptides inhibit macromolecular synthesis in *Escherichia coli*. Antimicrob. Agents Chemother. **46**, 605-614

73. Subbalakshmi, C. and Sitaram, N. (1998) Mechanism of antimicrobial action of indolicidin. FEMS Microbiol. Lett. **160**, 91-96

74. Park, C. B., Yi, K. S., Matsuzaki, K., Kim, M. S. and Kim, S. C. (2000) Structure-activity analysis of buforin II, a histone H2A-derived antimicrobial peptide: the proline hinge is responsible for the cell-penetrating ability of buforin II. Proc. Natl. Acad. Sci. U. S. A. **97**, 8245-8250

75. Park, C. B., Kim, H. S. and Kim, S. C. (1998) Mechanism of action of the antimicrobial peptide buforin II: buforin II kills microorganisms by penetrating the cell membrane and inhibiting cellular functions. Biochem. Biophys. Res. Commun. **244**, 253-257

76. Lehrer, R. I., Barton, A., Daher, K. A., Harwig, S. S., Ganz, T. and Selsted, M. E. (1989) Interaction of human defensins with *Escherichia coli*. mechanism of bactericidal activity. J. Clin. Invest. **84**, 553-561

77. Kragol, G., Lovas, S., Varadi, G., Condie, B. A., Hoffmann, R. and Otvos, L. (2001) The antibacterial peptide pyrrhocoricin inhibits the ATPase actions of DnaK and prevents chaperone-assisted protein folding. Biochemistry. **40**, 3016-3026

78. Brumfitt, W., Salton, M. R. and Hamilton-Miller, J. M. (2002) Nisin, alone and combined with peptidoglycan-modulating antibiotics: activity against methicillin-resistant *Staphylococcus aureus* and vancomycin-resistant enterococci. J. Antimicrob. Chemother. **50**, 731-734

79. Brötz, H., Bierbaum, G., Reynolds, P. E. and Sahl, H. (1997) The lantibiotic mersacidin inhibits peptidoglycan biosynthesis at the level of transglycosylation. Eur. J. Biochem. **246**, 193-199

80. Peschel, A. and Sahl, H. G. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4**, 529-536

81. Trybala, E., Olofsson, S., Mårdberg, K., Svennerholm, B., Umemoto, K., Glorioso, J. C. and Bergström, T. (2004) Structural and functional features of the polycationic peptide required for inhibition of herpes simplex virus invasion of cells. Antiviral Res. **62**, 125-134

82. Spillmann, D. (2001) Heparan sulfate: anchor for viral intruders?. Biochimie. **83**, 811-817

83. Jenssen, H., Andersen, J. H., Uhlin-Hansen, L., Gutteberg, T. J. and Rekdal, Ø. (2004) Anti-HSV activity of lactoferricin analogues is only partly related to their affinity for heparan sulfate. Antiviral Res. **61**, 101-109

84. Tamamura, H., Xu, Y., Hattori, T., Zhang, X., Arakaki, R., Kanbara, K., Omagari, A., Otaka, A., Ibuka, T., Yamamoto, N., Nakashima, H. and Fujii, N. (1998) A low-molecular-weight inhibitor against the chemokine receptor CXCR4: a strong anti-HIV peptide T140. Biochem. Biophys. Res. Commun. **253**, 877-882

85. De Lucca, A. J. and Walsh, T. J. (1999) Antifungal peptides: novel therapeutic compounds against emerging pathogens. Antimicrob. Agents Chemother. **43**, 1-11

86. Lorin, C., Saidi, H., Belaid, A., Zairi, A., Baleux, F., Hocini, H., Bélec, L., Hani, K. and Tangy, F. (2005) The antimicrobial peptide dermaseptin S4 inhibits HIV-1 infectivity *in vitro*. Virology. **334**, 264-275

87. Murakami, T., Niwa, M., Tokunaga, F., Miyata, T. and Iwanaga, S. (1991) Direct virus inactivation of tachyplesin I and its isopeptides from horseshoe crab hemocytes. Chemotherapy. **37**, 327-334

88. Jenssen, H., Andersen, J. H., Mantzilas, D. and Gutteberg, T. J. (2004) A wide range of medium-sized, highly cationic, α-helical peptides show antiviral activity against herpes simplex virus. Antiviral Res. **64**, 119-126

89. Yasin, B., Wang, W., Pang, M., Cheshenko, N., Hong, T., Waring, A. J., Herold, B. C., Wagar, E. A. and Lehrer, R. I. (2004) Theta defensins protect cells from infection by herpes simplex virus by inhibiting viral adhesion and entry. J. Virol. **78**, 5147-5156

90. Thevissen, K., Ghazi, A., De Samblanx, G. W., Brownlee, C., Osborn, R. W. and Broekaert, W. F. (1996) Fungal membrane responses induced by plant defensins and thionins. J. Biol. Chem. **271**, 15018-15025

91. Larrañaga, P., Díaz-Dellavalle, P., Cabrera, A., Alem, D., Leoni, C., Souza, A. L. A., Giovanni-De-Simone, S. and Dalla-Rizza, M. (2012) Activity of naturally derived antimicrobial peptides against filamentous fungi relevant for agriculture. Sus. Agric. Res. **1**, 211-221

92. Koo, J. C., Lee, B., Young, M. E., Koo, S. C., Cooper, J. A., Baek, D., Lim, C. O., Lee, S. Y., Yun, D. J. and Cho, M. J. (2004) Pn-AMP1, a plant defense protein, induces actin depolarization in yeasts. Plant Cell Physiol. **45**, 1669-1680

93. Madeo, F., Carmona-Gutierrez, D., Ring, J., Büttner, S., Eisenberg, T. and Kroemer, G. (2009) Caspase-dependent and caspase-independent cell death pathways in yeast. Biochem. Biophys. Res. Commun. **382**, 227-231

94. Narasimhan, M. L., Damsz, B., Coca, M. A., Ibeas, J. I., Yun, D. J., Pardo, J. M., Hasegawa, P. M. and Bressan, R. A. (2001) A plant defense response effector induces microbial apoptosis. Mol. Cell. **8**, 921-930

95. Aerts, A. M., Francois, I. E., Meert, E. M., Li, Q. T., Cammue, B. P. and Thevissen, K. (2007) The antifungal activity of RsAFP2, a plant defensin from *Raphanus sativus*, involves the induction of reactive oxygen species in *Candida albicans*. J. Mol. Microbiol. Biotechnol. **13**, 243-247

96. Munoz, A., Lopez-Garcia, B. and Marcos, J. F. (2006) Studies on the mode of action of the antifungal hexapeptide PAF26. Antimicrob. Agents Chemother. **50**, 3847-3855

97. Troskie, A. M., de Beer, A., Vosloo, J. A., Jacobs, K. and Rautenbach, M. (2014) Inhibition of agronomically relevant fungal phytopathogens by tyrocidines, cyclic antimicrobial peptides isolated from *Bacillus aneurinolyticus*. Microbiology. **160**, 2089-2101

98. Troskie, A. M. (2014) Tyrocidines, cyclic decapeptides produced by soil bacilli, as potent inhibitors of fungal pathogens. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/86162

99. Zasloff, M. (1987) Magainins, a class of antimicrobial peptides from *Xenopus skin*: isolation, characterization of two active forms, and partial cDNA sequence of a precursor. Proc. Natl. Acad. Sci. U. S. A. **84**, 5449-5453

100. McGwire, B. S., Olson, C. L., Tack, B. F. and Engman, D. M. (2003) Killing of african trypanosomes by antimicrobial peptides. J. Infect. Dis. **188**, 146-152

101. Park, Y., Jang, S., Lee, D. G. and Hahm, K. (2004) Antinematodal effect of antimicrobial peptide, PMAP-23, isolated from porcine myeloid against *Caenorhabditis elegans*. J. Pept. Sci. **10**, 304-311

102. Luque-Ortega, J. R., van't Hof, W., Veerman, E. C. I., Saugar, J. M. and Rivas, L. (2008) Human antimicrobial peptide histatin 5 is a cell-penetrating peptide targeting mitochondrial ATP synthesis in *Leishmania*. FASEB J. **22**, 1817-1828

103. Gwadz, R. W., Kaslow, D., Lee, J. Y., Maloy, W. L., Zasloff, M. and Miller, L. H. (1989) Effects of magainins and cecropins on the sporogonic development of malaria parasites in mosquitoes. Infect. Immun. **57**, 2628-2633

104. Rautenbach, M., Vlok, N. M., Stander, M. and Hoppe, H. C. (2007) Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from *Bacillus brevis*. Biochim. Biophys. Acta. **1768**, 1488-1497

105. Shahabuddin, M., Fields, I., Bulet, P., Hoffmann, J. A. and Miller, L. H. (1998) *Plasmodium gallinaceum*: differential killing of some mosquito stages of the parasite by insect defensin. Exp. Parasitol. **89**, 103-112

106. Yeaman, M. R. and Yount, N. Y. (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. **55**, 27-55

107. Steinberg, D. A., Hurst, M. A., Fujii, C. A., Kung, A. H., Ho, J. F., Cheng, F. C., Loury, D. J. and Fiddes, J. C. (1997) Protegrin-1: a broad-spectrum, rapidly microbicidal peptide with *in vivo* activity. Antimicrob. Agents Chemother. **41**, 1738-1742

108. Peschel, A., Otto, M., Jack, R. W., Kalbacher, H., Jung, G. and Gotz, F. (1999) Inactivation of the *dlt* operon in *Staphylococcus aureus* confers sensitivity to defensins, protegrins, and other antimicrobial peptides. J. Biol. Chem. **274**, 8405-8410

109. Peschel, A., Jack, R. W., Otto, M., Collins, L. V., Staubitz, P., Nicholson, G., Kalbacher, H., Nieuwenhuizen, W. F., Jung, G., Tarkowski, A., van Kessel, K. P. and van Strijp, J. A. (2001) *Staphylococcus aureus* resistance to human defensins and evasion of neutrophil killing via the novel virulence factor MprF is based on modification of membrane lipids with L-lysine. J. Exp. Med. **193**, 1067-1076

110. Gunn, J. S., Lim, K. B., Krueger, J., Kim, K., Guo, L., Hackett, M. and Miller, S. I. (1998) PmrA-PmrB-regulated genes necessary for 4-aminoarabinose lipid A modification and polymyxin resistance. Mol. Microbiol. **27**, 1171-1182

111. Guo, L., Lim, K. B., Poduje, C. M., Daniel, M., Gunn, J. S., Hackett, M. and Miller, S. I. (1998) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell. **95**, 189-198

112. Visser, L. G., Hiemstra, P. S., van den Barselaar, M. T., Ballieux, P. A. and van Furth, R. (1996) Role of YadA in resistance to killing of *Yersinia enterocolitica* by antimicrobial polypeptides of human granulocytes. Infect. Immun. **64**, 1653-1658

113. Roland, K. L., Esther, C. R. and Spitznagel, J. K. (1994) Isolation and characterization of a gene, *pmrD*, from *Salmonella typhimurium* that confers resistance to polymyxin when expressed in multiple copies. J. Bacteriol. **176**, 3589-3597

114. Nikaido, H. (1996) Multidrug efflux pumps of Gram-negative bacteria. J. Bacteriol. **178**, 5853-5859

115. Tzeng, Y. L., Ambrose, K. D., Zughaier, S., Zhou, X., Miller, Y. K., Shafer, W. M. and Stephens, D. S. (2005) Cationic antimicrobial peptide resistance in *Neisseria meningitidis*. J. Bacteriol. **187**, 5387-5396

116. Bengoechea, J. A. and Skurnik, M. (2000) Temperature-regulated efflux pump/potassium antiporter system mediates resistance to cationic antimicrobial peptides in *Yersinia*. Mol. Microbiol. **37**, 67-80

117. Groisman, E. A. (1994) How bacteria resist killing by host-defense peptides. Trends Microbiol. **2**, 444-449

118. Burmolle, M., Webb, J. S., Rao, D., Hansen, L. H., Sorensen, S. J. and Kjelleberg, S. (2006) Enhanced biofilm formation and increased resistance to antimicrobial agents and bacterial invasion are caused by synergistic interactions in multispecies biofilms. Appl. Environ. Microbiol. **72**, 3916-3923

119. Falagas, M. E. and Kasiakou, S. K. (2005) Colistin: the revival of polymyxins for the management of multidrug-resistant Gram-negative bacterial infections. Clin. Infect. Dis. **40**, 1333-1341

120. Gause, G. F. and Brazhnikova, M. G. (1944) Gramicidin S and its use in the treatment of infected wounds. Nature. **154**, 703

121. Werner, A. H. and Russell, A. D. (1999) Mupirocin, fusidic acid and bacitracin: activity, action and clinical uses of three topical antibiotics. Vet. Dermatol. **10**, 225-240

122. Steenbergen, J. N., Alder, J., Thorne, G. M. and Tally, F. P. (2005) Daptomycin: a lipopeptide antibiotic for the treatment of serious Gram-positive infections. J. Antimicrob. Chemother. **55**, 283-288

123. Cheigh, C. I. and Pyun, Y. R. (2005) Nisin biosynthesis and its properties. Biotechnol. Lett. **27**, 1641-1648

124. McInturff, J. E., Wang, S. J., Machleidt, T., Lin, T. R., Oren, A., Hertz, C. J., Krutzik, S. R., Hart, S., Zeh, K., Anderson, D. H., Gallo, R. L., Modlin, R. L. and Kim, J. (2005) Granulysin-derived peptides demonstrate antimicrobial and anti-inflammatory effects against *Propionibacterium acnes*. J. Invest. Dermatol. **125**, 256-263

125. Pfeufer, N. Y., Hofmann-Peiker, K., Hofmann-Peiker, K., Warnke, P. H., Weigel, M. C. and Kleine, M. (2011) Bioactive coating of titanium surfaces with recombinant human *β*-defensin-2 (rHu*β*D2) may prevent bacterial colonization in orthopaedic surgery. J. Bone Joint Surg. Am. **93**, 840-846

126. Cook, R. J., Bruckart, W. L., Coulson, J. R., Goettel, M. S., Humber, R. A., Lumsden, R. D., Maddox, J. V., McManus, M. L., Moore, L., Meyer, S. F., Quimby, P. C., Stack, J. P., Jr. and Vaughn, J. L. (1996) Safety of microorganisms intended for pest and plant disease control: a framework for scientific evaluation. Biol. Control. **7**, 333-351

127. Compant, S., Duffy, B., Nowak, J., Clement, C. and Barka, E. A. (2005) Use of plant growth-promoting bacteria for biocontrol of plant diseases: principles, mechanisms of action, and future prospects. Appl. Environ. Microbiol. **71**, 4951-4959

128. Weller, D. M. (1988) Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. **26**, 379-407

129. Sardi, P., Saracchi, M., Quaroni, S., Petrolini, B., Borgonovi, G. E. and Merli, S. (1992) Isolation of endophytic *Streptomyces* strains from surface-sterilized roots. Appl. Environ. Microbiol. **58**, 2691-2693

130. Montesinos, E. (2007) Antimicrobial peptides and plant disease control. FEMS Microbiol. Lett. **270**, 1-11

131. Danders, W., Marahiel, M. A., Krause, M., Kosui, N., Kato, T., Izumiya, N. and Kleinkauf, H. (1982) Antibacterial action of gramicidin S and tyrocidines in relation to active transport, *in vitro* transcription, and spore outgrowth. Antimicrob. Agents Chemother. **22**, 785-790

132. Izumiya, N., Kato, T., Aoyagi, H., Waki, M. and Kondo, M. (1979) Synthetic aspects of biologically active cyclic peptides: gramicidin S and tyrocidines. Halsted Press, New York, USA.

133. Kondejewski, L. H., Farmer, S. W., Wishart, D. S., Kay, C. M., Hancock, R. E. W. and Hodges, R. S. (1996) Modulation of structure and antibacterial and hemolytic activity by ring size in cyclic gramicidin S analogs. J. Biol. Chem. **271**, 25261-25268

134. McPhee, J. B., Scott, M. G. and Hancock, R. E. W. (2005) Design of host defence peptides for antimicrobial and immunity enhancing activities. Comb. Chem. High Throughput Screen. **8**, 257-272

135. Masip, I., Perez-Paya, E. and Messeguer, A. (2005) Peptoids as source of compounds eliciting antibacterial activity. Comb. Chem. High Throughput Screen. **8**, 235-239

136. Freidinger, R. M. (2003) Design and synthesis of novel bioactive peptides and peptidomimetics. J. Med. Chem. **46**, 5553-5566

137. Gould, K. (2016) Antibiotics: from prehistory to the present day. J. Antimicrob. Chemother. **71**, 572-575

138. Foster, R. (1988) Microenvironments of soil microorganisms. Biol. Fertil. Soils. **6**, 189-203

139. Ranjard, L. and Richaume, A. (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res. Microbiol. **152**, 707-716

140. Daniel, R. (2004) The soil metagenome-a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. **15**, 199-204

141. Hibbing, M. E., Fuqua, C., Parsek, M. R. and Peterson, S. B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. **8**, 15-25

142. Maróti, G., Kereszt, A., Kondorosi, É and Mergaert, P. (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. Res. Microbiol. **162**, 363-374

143. Riley, M. A. and Gordon, D. M. (1999) The ecological role of bacteriocins in bacterial competition. Trends Microbiol. **7**, 129-133

144. Greer, G. G. and Dilts, B. D. (1995) Lactic acid inhibition of the growth of spoilage bacteria and cold tolerant pathogens on pork. Int. J. Food Microbiol. **25**, 141-151

145. Adams, M. R. and Hall, C. J. (1988) Growth inhibition of food-borne pathogens by lactic and acetic acids and their mixtures. Int. J. Food Sci. Technol. **23**, 287-292

146. Kerr, B., Riley, M. A., Feldman, M. W. and Bohannan, B. J. (2002) Local dispersal promotes biodiversity in a real-life game of rock-paper-scissors. Nature. **418**, 171-174

147. Boles, B. R., Thoendel, M. and Singh, P. K. (2004) Self-generated diversity produces "insurance effects" in biofilm communities. Proc. Natl. Acad. Sci. U. S. A. **101**, 16630-16635

148. Czárán, T. L., Hoekstra, R. F. and Pagie, L. (2002) Chemical warfare between microbes promotes biodiversity. Proc. Natl. Acad. Sci. U. S. A. **99**, 786-790

149. Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. **25**, 39-67

150. Fleming, A. (1929) On the antibacterial action of cultures of a *Penicillium*, with special reference to their use in the isolation of *B. influenzae*. Br. J. Exp. Pathol. **10**, 226-236

151. Chain, E., Florey, H. W., Adelaide, M. B., Gardner, A. D., Heatley, N. G., Jennings, M. A., Orr-Ewing, J. and Sanders, A. G. (1940) Penicillin as a chemotherapeutic agent. Lancet. **236**, 226-228

152. Hotchkiss, R. D. and Dubos, R. J. (1940) Fractionation of the bactericidal agent from cultures of a soil *Bacillus*. J. Biol. Chem. **132**, 791-792

153. Dubos, R. J. and Hotchkiss, R. D. (1941) The production of bactericidal substances by aerobic sporulating bacilli. J. Exp. Med. **73**, 629-640

154. Maycock, A. L., Chang, A., Farrar, J. J. and Balogh, I. Adolor Corporation. (1998) Spray formulations of antihyperalgesic opiates and method of treating topical hyperalgesic conditions therewith. **U.S. Patent** 5811078

155. Schatz, A., Bugle, E. and Waksman, S. A. (1944) Streptomycin, a substance exhibiting antibiotic activity against Gram-positive and Gram-negative bacteria. Proc. Soc. Exp. Biol. Med. **55**, 66-69

156. Craik, D. J., Fairlie, D. P., Liras, S. and Price, D. (2013) The future of peptide-based drugs. Chem. Biol. Drug Des. **81**, 136-147

157. Rebuffat, S. (2011) Bacteriocins from Gram-negative bacteria: A classification? In Prokaryotic antimicrobial peptides: from genes to applications (Drider, D. and Rebuffat, S., eds.), pp. 55-72, Springer, New York, USA.

158. Riley, M. A. and Chavan, M. A. (2007) Molecular evolution of bacteriocins in Gram-negative bacteria. In Bacteriocins: ecology and evolution (Riley, M. A. and Chavan, M. A., eds.), pp. 19-43, Springer, Berlin, Germany

159. Asensio, C., Pérez-Díaz, J. C., Martínez, M. C. and Baquero, F. (1976) A new family of low molecular weight antibiotics from enterobacteria. Biochem. Biophys. Res. Commun. **69**, 7-14

160. Gillor, O., Kirkup, B. C. and Riley, M. A. (2004) Colicins and microcins: the next generation antimicrobials. Adv. Appl. Microbiol. **54**, 129-146

161. Gordon, D. M. and O'Brien, C. L. (2006) Bacteriocin diversity and the frequency of multiple bacteriocin production in *Escherichia coli*. Microbiology. **152**, 3239-3244

162. Cascales, E., Buchanan, S. K., Duche, D., Kleanthous, C., Lloubes, R., Postle, K., Riley, M., Slatin, S. and Cavard, D. (2007) Colicin biology. Microbiol. Mol. Biol. Rev. **71**, 158-229

163. Elkins, P., Bunker, A., Cramer, W. A. and Stauffacher, C. V. (1997) A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1. Structure. **5**, 443-458

164. Vetter, I. R., Parker, M. W., Tucker, A. D., Lakey, J. H., Pattus, F. and Tsernoglou, D. (1998) Crystal structure of a colicin N fragment suggests a model for toxicity. Structure. **6**, 863-874

165. Guihard, G., Benedetti, H., Besnard, M. and Letellier, L. (1993) Phosphate efflux through the channels formed by colicins and phage T5 in *Escherichia coli* cells is responsible for the fall in cytoplasmic ATP. J. Biol. Chem. **268**, 17775-17780

166. Harkness, R. E. and Braun, V. (1989) Inhibition of lipopolysaccharide O-antigen synthesis by colicin M. J. Biol. Chem. **264**, 14716-14722

167. Garinot-Schneider, C., Pommer, A. J., Moore, G. R., Kleanthous, C. and James, R. (1996) Identification of putative active-site residues in the DNase domain of colicin E9 by random mutagenesis. J. Mol. Biol. **260**, 731-742

168. Lagos, R., Wilkens, M., Vergara, C., Cecchi, X. and Monasterio, O. (1993) Microcin E492 forms ion channels in phospholipid bilayer membranes. FEBS Lett. **321**, 145-148

169. Guijarro, J. I., Gonzalez-Pastor, J. E., Baleux, F., San Millan, J. L., Castilla, M. A., Rico, M., Moreno, F. and Delepierre, M. (1995) Chemical structure and translation inhibition studies of the antibiotic microcin C7. J. Biol. Chem. **270**, 23520-23532

170. Martinez, J. L. and Perez-Diaz, J. C. (1986) Isolation, characterization, and mode of action on *Escherichia coli* strains of microcin D93. Antimicrob. Agents Chemother. **29**, 456-460

171. Salomon, R. A. and Farias, R. N. (1992) Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J. Bacteriol. **174**, 7428-7435

172. Wilson, M. (2005) The gastrointestinal tract and its indigenous microbiota. In Microbial inhabitants of humans: their ecology and role in health and disease.(Anonymous), pp. 251-317, University of Cambridge Press, Cambridge, UK

173. Estrada, I. B., Aller, A., Aller, F., Gómez, X. and Morán, A. (2004) The survival of *Escherichia coli*, faecal coliforms and enterobacteriaceae in general in soil treated with sludge from wastewater treatment plants. Bioresour. Technol. **93**, 191-198

174. Heng, N. C. K., Wescombe, P. A., Burton, J. P., Jack, R. W. and Tagg, J. R. (2007) The diversity of bacteriocins in Gram-positive bacteria. In Bacteriocins: ecology and evolution (Riley, M. A. and Chavan, M. A., eds.), pp. 45-92, Springer, Berlin, Germany

175. Klaenhammer, T. R. (1993) Genetics of bacteriocins produced by lactic acid bacteria. FEMS Microbiol. Rev. **12**, 39-85

176. Chatterjee, C., Paul, M., Xie, L. and van der Donk, Wilfred A. (2005) Biosynthesis and mode of action of lantibiotics. Chem. Rev. **105**, 633-684

177. Morell, J. L. and Gross, E. (1973) Configuration of the β-carbon atoms of the β-methyllanthionine residues in nisin. J. Am. Chem. Soc. **95**, 6480-6481

178. Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B. and Sahl, H. G. (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276**, 1772-1779

179. Thennarasu, S., Lee, D. K., Poon, A., Kawulka, K. E., Vederas, J. C. and Ramamoorthy, A. (2005) Membrane permeabilization, orientation, and antimicrobial mechanism of subtilosin A. Chem. Phys. Lipids. **137**, 38-51

180. Gut, I. M., Blanke, S. R. and van der Donk, Wilfred A. (2011) Mechanism of inhibition of *Bacillus anthracis* spore outgrowth by the lantibiotic nisin. ACS Chem. Biol. **6**, 744-752

181. Rogers, L. A. and Whittier, E. O. (1928) Limiting factors in the lactic fermentation. J. Bacteriol. **16**, 211-229

182. Mattick, A., Hirsch, A. and Berridge, N. (1947) Further observations on an inhibitory substance (nisin) from lactic streptococci. Lancet. **250**, 5-8

183. Deegan, L. H., Cotter, P. D., Hill, C. and Ross, P. (2006) Bacteriocins: biological tools for bio-preservation and shelf-life extension. Int. Dairy J. **16**, 1058-1071

184. Rea, M. C., Ross, R. P., Cotter, P. D. and Hill, C. (2011) Classification of bacteriocins from Gram-positive bacteria. In Prokaryotic antimicrobial peptides-from genes to applications (Drider, D. and Rebuffat, S., eds.), pp. 29-53, Springer, New York, USA.

185. Nissen-Meyer, J., Rogne, P., Oppegard, C., Haugen, H. S. and Kristiansen, P. E. (2009) Structure-function relationships of the non-lanthionine-containing peptide (class II) bacteriocins produced by Gram-positive bacteria. Curr. Pharm. Biotechnol. **10**, 19-37

186. O'sullivan, L., Ross, R. P. and Hill, C. (2002) Potential of bacteriocin-producing lactic acid bacteria for improvements in food safety and quality. Biochimie. **84**, 593-604

187. Chen, Y. S., Yanagida, F. and Shinohara, T. (2005) Isolation and identification of lactic acid bacteria from soil using an enrichment procedure. Lett. Appl. Microbiol. **40**, 195-200

188. Sumi, C. D., Yang, B. W., Yeo, I. C. and Hahm, Y. T. (2015) Antimicrobial peptides of the genus *Bacillus*: a new era for antibiotics. Can. J. Microbiol. **61**, 93-103

189. Aunpad, R. and Na-Bangchang, K. (2007) Pumilicin 4, a novel bacteriocin with anti-MRSA and anti-VRE activity produced by newly isolated bacteria *Bacillus pumilus* strain WAPB4. Curr. Microbiol. **55**, 308-313

190. Wang, J., Zhang, L., Teng, K., Sun, S., Sun, Z. and Zhong, J. (2014) Cerecidins, novel lantibiotics from *Bacillus cereus* with potent antimicrobial activity. Appl. Environ. Microbiol. **80**, 2633-2643

191. Foster, J. W. and Woodruff, H. B. (1946) Bacillin, a new antibiotic substance from a soil isolate of *Bacillus subtilis*. J. Bacteriol. **51**, 363-369

192. Babasaki, K., Takao, T., Shimonishi, Y. and Kurahashi, K. (1985) Subtilosin A, a new antibiotic peptide produced by *Bacillus subtilis* 168: isolation, structural analysis, and biogenesis. J. Biochem. **98**, 585-603

193. Arguelles Arias, A., Ongena, M., Devreese, B., Terrak, M., Joris, B. and Fickers, P. (2013) Characterization of amylolysin, a novel lantibiotic from *Bacillus amyloliquefaciens* GA1. PLos One. **8**, e83037. 10.1371/journal.pone.0083037

194. Hyronimus, B., Le Marrec, C. and Urdaci, M. C. (1998) Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulans* I_4. J. Appl. Microbiol. **85**, 42-50

195. Holland, I. B. and Roberts, C. F. (1964) Some properties of a new bacteriocin formed by *Bacillus megaterium*. J. Gen. Microbiol. **35**, 271-285

196. Cantú, T. G., Yamanaka-Yuen, N. A. and Lietman, P. S. (1994) Serum vancomycin concentrations: reappraisal of their clinical value. Clin. Infect. Dis. **18**, 533-543

197. Froudarakis, M., Hatzimichael, E., Kyriazopoulou, L., Lagos, K., Pappas, P., Tzakos, A. G., Karavasilis, V., Daliani, D., Papandreou, C. and Briasoulis, E. (2013) Revisiting bleomycin from pathophysiology to safe clinical use. Crit. Rev. Oncol. Hematol. **87**, 90-100

198. Singh, P. and Cameotra, S. S. (2004) Potential applications of microbial surfactants in biomedical sciences. Trends Biotechnol. **22**, 142-146

199. Maget-Dana, R. and Peypoux, F. (1994) Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology. **87**, 151-174

200. Tang, X., Thibault, P. and Boyd, R. K. (1992) Characterisation of the tyrocidine and gramicidin fractions of the tyrothricin complex from *Bacillus brevis* using liquid chromatography and mass spectrometry. Int. J. Mass Spectrom. Ion Processes. **122**, 153-179

201. Mootz, H. D. and Marahiel, M. A. (1997) The tyrocidine biosynthesis operon of *Bacillus brevis*: complete nucleotide sequence and biochemical characterization of functional internal adenylation domains. J. Bacteriol. **179**, 6843-6850

202. Munyuki, G., Jackson, G. E., Venter, G. A., Kövér, K. E., Szilágyi, L., Rautenbach, M., Spathelf, B. M., Bhattacharya, B. and van der Spoel, D. (2013) *β*-sheet structures and dimer models of the two major tyrocidines, antimicrobial peptides from *Bacillus aneurinolyticus*. Biochemistry. **52**, 7798-7806

203. Kuo, M. C. and Gibbons, W. A. (1979) Determination of individual side-chain conformations, tertiary conformations, and molecular topography of tyrocidine A from scalar coupling constants and chemical shifts. Biochemistry. **18**, 5855-5867

204. Spathelf, B. M. and Rautenbach, M. (2009) Anti-listerial activity and structure-activity relationships of the six major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Bioorg. Med. Chem. **17**, 5541-5548

205. Leussa, A. N. (2014) Characterisation of small cyclic peptides with antimalarial and antilisterial activity. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/86161

206. Spathelf, B. M. (2010) Qualitative structure-actvity relationships of the major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/4001

207. Aranda, F. J. and de Kruijff, B. (1988) Interrelationships between tyrocidine and gramicidin A′ in their interaction with phospholipids in model membranes. Biochim. Biophys. Acta. **937**, 195-203

208. Mach, B. and Slayman, C. W. (1966) Mode of action of tyrocidine on *Neurospora*. Biochim. Biophys. Acta. **124**, 351-361

209. Johnson, B. A., Anker, H. and Meleney, F. L. (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science. **102**, 376-377

210. Citron, D. M., Merriam, C. V., Tyrrell, K. L., Warren, Y. A., Fernandez, H. and Goldstein, E. J. C. (2003) *In vitro* activities of ramoplanin, teicoplanin, vancomycin, linezolid, bacitracin, and four other antimicrobials against intestinal anaerobic bacteria. Antimicrob. Agents Chemother. **47**, 2334-2338

211. Govaerts, C., Li, C., Orwa, J., Van Schepdael, A., Adams, E., Roets, E. and Hoogmartens, J. (2003) Sequencing of bacitracin A and related minor components by liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. Rapid Commun. Mass Spectrom. **17**, 1366-1379

212. Wagner, B., Schumann, D., Linne, U., Koert, U. and Marahiel, M. A. (2006) Rational design of bacitracin A derivatives by incorporating natural product derived heterocycles. J. Am. Chem. Soc. **128**, 10513-10520

213. Lyerla, J. R.,Jr and Freedman, M. H. (1972) Spectral assignment and conformational analysis of cyclic peptides by carbon-13 nuclear magnetic resonance. J. Biol. Chem. **247**, 8183-8192

214. Anderson, J. S., Matsuhashi, M., Haskin, M. A. and Strominger, J. L. (1967) Biosythesis of the peptidoglycan of bacterial cell walls. II. phospholipid carriers in the reaction sequence. J. Biol. Chem. **242**, 3180-3190

215. Bohnenberger, E. and Sandermann, H.,Jr. (1976) Dephosphorylation of C_{55}-isoprenyl-monophosphate by non-specific phosphatases. FEBS Lett. **67**, 85-89

216. Storm, D. R. and Strominger, J. L. (1974) Binding of bacitracin to cells and protoplasts of *Micrococcus lysodeikticus*. J. Biol. Chem. **249**, 1823-1827

217. Baltz, R. H. (2008) Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. **8**, 557-563

218. McCaig, A. E., Grayston, S. J., Prosser, J. I. and Glover, L. A. (2001) Impact of cultivation on characterisation of species composition of soil bacterial communities. FEMS Microbiol. Ecol. **35**, 37-48

219. Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N. N., Anderson, I. J., Cheng, J. F., Darling, A., Malfatti, S., Swan, B. K., Gies, E. A., Dodsworth, J. A., Hedlund, B. P., Tsiamis, G., Sievert, S. M., Liu, W. T., Eisen, J. A., Hallam, S. J., Kyrpides, N. C., Stepanauskas, R., Rubin, E. M., Hugenholtz, P. and Woyke, T. (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature. **499**, 431-437

220. Ling, L. L., Schneider, T., Peoples, A. J., Spoering, A. L., Engels, I., Conlon, B. P., Mueller, A., Schäberle, T. F., Hughes, D. E., Epstein, S., Jones, M., Lazarides, L., Steadman, V. A., Cohen, D. R., Felix, C. R., Fetterman, K. A., Millett, W. P., Nitti, A. G., Zullo, A. M., Chen, C. and Lewis, K. (2015) A new antibiotic kills pathogens without detectable resistance. Nature. **517**, 455-459

221. Nichols, D., Cahoon, N., Trakhtenberg, E. M., Pham, L., Mehta, A., Belanger, A., Kanigan, T., Lewis, K. and Epstein, S. S. (2010) Use of ichip for high-throughput *in situ* cultivation of "uncultivable" microbial species. Appl. Environ. Microbiol. **76**, 2445-2450

Chapter 2
Isolation and identification of antimicrobial producing bacteria from a commercial soil additive

2.1 Introduction

Antimicrobial peptides were among the first antimicrobial agents discovered, yet their development and use in the clinical sector were stunted. This was due to conventional antibiotics generally having higher antimicrobial activity and lower cytotoxicity than antimicrobial peptides [1]. However, increasing resistance of pathogenic microbes towards conventional antibiotics has sparked an interest and revived antimicrobial peptide research [2].

Most antimicrobial peptides elicit their bactericidal activity by disrupting bacterial cellular membranes [3,4]. Various antimicrobial peptides have also shown inhibition of intracellular processes such as cell wall synthesis, DNA synthesis and RNA synthesis in in addition to their membranolytic activity [3-6]. This multiple target mode of action of the majority of antimicrobial peptides significantly decreases the likelihood of resistance development relative to conventional antibiotics, which generally have a single, specific mode of action [7]. An example of an antimicrobial peptide with multiple modes of action is nisin [8]. In addition to its membrane pore forming activity, nisin can bind to the membrane-bound cell wall precursor, lipid II, inhibiting its incorporation into the peptidoglycan backbone resulting in the disruption of peptidoglycan synthesis [8,9]. Rapid bactericidal action, multiple cellular targets and low potential for pathogen resistance development has therefore increased interest in the development of antimicrobial peptides as a much needed, effective treatment for infectious diseases.

Antimicrobial peptides are crucial to the innate immune response of organisms throughout all kingdoms of life [10-14]. Historically, however, soil bacteria have shown to be an invaluable resource of a diverse array of antimicrobials including antimicrobial peptides [15]. Genomic studies have shown the soil biome to be one of the most diverse habitats in terms of bacterial species [16]. However, only a small percentage of these bacteria have been isolated and subjected to antimicrobial screening [17]. The immense diversity and historical success of soil bacteria antimicrobials makes it an ideal source for novel antimicrobial peptide screening.

Although advantageous, the immense diversity of soil bacteria also leads to several complications. The main obstacle is that the vast variety of bacterial strains present in soil, results in cumbersome screening methodologies. For this reason, this study first focused on validating a screening, isolation and purification methodology for obtaining novel antimicrobial peptides from a narrow spectrum of bacterial strains present in an undisclosed commercial soil additive. The soil additive is a microbial fertilizer used on agricultural crops and is comprised of three undisclosed soil *Bacillus* spp., as per the manufacturer's description. It is commonly used in conjunction with other fertilizers and is claimed to increase mineral uptake, promote the production of plant hormones, as well as suppress common plant root diseases. The latter claim directed us to hypothesise that at least one of the bacterial strains in the additive produces an antimicrobial agent.

This study focused on the isolation of bacterial strains from the commercial soil additive using traditional microbiological techniques. Morphologically different bacterial colonies were selected and screened for their antimicrobial and haemolytic activity. Furthermore, the study resulted in the development of an electrospray mass spectrometry (ESMS) method used to rapidly identify low molecular mass (M_r) compounds produced by bacterial isolates. This technique was developed to accelerate the screening process for novel low M_r antimicrobials,

including antimicrobial peptides, by eliminating the rediscovery of previously characterised antimicrobials based on their M_r. Isolates selected based on favourable anti-bacterial activity, haemolytic activity and low M_r ESMS spectra, were subjected to bacterial identification by both matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) biotyping and 16S rRNA gene sequencing.

2.2 Materials

2.2.1 Bacterial strains

Cultures of *Brevibacillus parabrevis* ATCC 10068 and *Micrococcus luteus* NCTC 8340 were supplied by the American Type Culture Collection (Manassas, VA, USA) and National Collection of Type Cultures (Proton Down, Salisbury, United Kingdom) respectively.

2.2.2 Research materials

The commercial soil additive was obtained from excess soil additive bottles retrieved from a grape farm. Nutrient broth (NB), tryptone soy broth (TSB), agar and the components for Luria Bertani broth (LB); yeast extract, tryptone and sodium chloride (NaCl) were obtained from Merck (Darmstadt, Germany). The Western Cape Blood services (National Health Laboratory, South Africa) supplied blood from anonymous A+ donors (300 mL enriched erythrocyte fraction containing a 100 mL of saline-adenine-glucose-mannitol red blood cell preservation solution and 63 mL citrate-phosphate-dextrose anticoagulant) conforming to relevant legislation and ethical guidelines. RPMI-1640 medium, hydrozyethyl piperazineethanesulfonic acid (HEPES), sodium bicarbonate, hypoxanthine, gentamicin, trifluoroacetic acid (TFA; >98%) and α-cyano-4-hydroxycinnamic acid (α- CHCA) MALDI-TOF matrix were obtained from Sigma Aldrich (St Louis, USA). Life Technologies (Melbourne, Australia) supplied Albumax II^{TM}. Petri dishes (90 mm), 50 mL polypropylene centrifuge tubes and 1.5 mL microfuge tubes were from Lasec (Cape Town, South Africa).

ZR Fungal/bacterial DNA kit and 2X KapaTaq Readymix were obtained from Zymo Research (California, USA) and KapaBiosystems (Cape Town, South Africa) respectively. Acetonitrile (ACN) (HPLC-grade, far UV cut-off) was obtained from Romill Ltd. (Cambridge, United Kingdom). Analytical grade water was prepared by filtering water from a reverse osmosis plant through a Millipore-Q® water purification system (Milford, USA).

2.3 Methods

2.3.1 Isolation of bacteria from a commercial soil additive

Bacterial colonies where isolated from two different bottles of commercial soil additive using standard microbiological techniques. First, spread plates where made with 100 µL of the two soil additive samples on agar (1.5% agar) plates containing different nutrient media including: TSB (30 g/L TSB), NB (16 g/L NB) and LB (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl). After 48 hours of incubation at 37°C, single colonies where selected based on visual colony morphological differences including: colour, form, margin, elevation, size, texture and appearance. Morphologically different colonies where streaked out onto respective culture media and incubated for 48 hours at 37°C. A single colony was subsequently selected and streaked onto both LB and TSB agar. After 48 hours of incubation at 37°C, the plates were stored at 4°C for less than a week, until identification of antimicrobial (section 2.3.2) and haemolytic activity (section 2.3.3). Single colonies from 4°C stock plates where freshly streaked onto both LB and TSB agar and incubated for 48 hours at 37°C, before antimicrobial and haemolytic activity analyses were performed.

2.3.2 Identification of antimicrobial producing isolates

Simultaneous antagonism spot-on-lawn analyses adapted from Tagg *et al*. [18] and Du Toit & Rautenbach [19] were performed to determine the production of antimicrobial compounds with activity towards the Gram-positive indicator *M. luteus*. A freezer stock of *M. luteus* was

streaked onto LB agar plates and incubated for 48 hours at 37°C. A single colony of *M. luteus* was then inoculated into 20 mL of LB broth and incubated overnight at 37°C with constant aeration. A sub-culture was made by the addition of 0.5 mL of the *M. luteus* starter culture in 20 mL of LB broth and incubated at 37°C until an optical density of 0.6 at 620 nm. The culture was diluted 1:10 in sterile LB agarose (LB broth containing 1% agarose) at ≤ 50°C and poured into culture dishes. After one hour, isolated bacterial colonies were transferred onto the lawn with a sterile pipette tip. *Br. parabrevis*, which produces the antimicrobial peptide tyrocidine, was used as a positive control. After 48 hours of incubation at 37°C the plates were inspected for the formation of clear inhibition zones surrounding bacterial colonies. These inhibition zones suggest that the bacterial colonies produce an antimicrobial agent that inhibits the growth of *M. luteus*. Photos of the spot plates were taken with a ChromaDoc-It TLC imaging system (UVP, California, USA) connected to a Canon Eos Rebel T3 digital camera (Canon, Tokyo, Japan). The inhibition zone size of isolate colonies were then compared to that of *Br. parabrevis* (the positive control) and *M. luteus* (negative control).

2.3.3 Preliminary determination of toxicity

Blood plate analyses were performed to determine whether the isolates produce beta-haemolytic compounds. Beta-hemolysis refers to the complete lysis of the erythrocyte cellular membrane. Blood (20 mL) was washed three times with 30 mL of RPMI-1640 media (10.4 g/L RPMI-1640, 4g/L glucose, 6 g/L HEPES, 2.1 g/L sodium bicarbonate, 5 g/L Albumax II^{TM}, 0.4 g/L hypoxanthine and 50 mg/L gentamicin) by centrifugation at 1200×*g* for 6 minutes per wash, and the plasma aspirated. The washed blood was subsequently stored at 4°C. Washed blood was diluted to 2% haematocrit in TSB agarose (TSB broth containing 1% agarose) at 50°C and poured into petri dishes. After one hour, isolates were transferred onto the blood plates using a sterile pipette tip. *Br. parabrevis* produce highly haemolytic

antimicrobial peptides, namely the tyrocidines and their analogues, and was therefore used as a positive control. The same imaging system as described in section 2.3.2 was used to take photos of the spot plates after 24 and 48 hours of incubation. The spot plates were subsequently inspected for the presence of clear zones surrounding bacterial colonies, which is indicative of beta-haemolysis. The size of clear zones around isolate colonies were then compared to those of *Br. parabrevis* (positive control) and *M. luteus* (negative control).

2.3.4 MALDI-MS analysis of low M_r compounds produced by selected isolates

The selected isolates and *Br. parabrevis* (positive control) were streaked onto TSB agar plates and incubated for 72 hours at 37°C. For MALDI-MS analysis a thin film of each microorganism culture was deposited onto a 384 MALDI polished steel target plate (in triplicate) using a direct transfer method and allowed to air dry. The dried sample was then mixed with 1 µL of α-CHCA matrix (10 mg/mL in 50% ACN; 2.5% TFA) and allowed to air dry. Samples were subsequently subjected low M_r profile analysis with an UltrafleXtreme MALDI TOF/TOF (Bruker Daltonics, Bremen, Germany) mass spectrometer. For low M_r spectra (peptide spectrum), the linear positive mode was used to acquire spectra within a *m/z* range of 500 to 3000. The initial laser power was set to 51% to obtain peaks. Spectrum acquisition was performed manually using the software, FlexControl version 3.4 (Bruker Daltonics, Bremen, Germany), by rastering the target spot 7 times with accumulated peptide spectra of 2100 shots, with a laser frequency of 1000 Hz. MALDI-MS experiments were conducted at the Proteomics Research & Services Unit, Department of Biotechnology, University of the Western Cape, South Africa.

2.3.5 ESMS analysis of low M_r compounds produced by selected isolates

A time-of-flight (TOF) ESMS method was developed to determine the small molecule profile of the selected soil additive isolates. The selected isolates and *Br. parabrevis* (positive

control) colonies where directly inoculated into microfuge tubes (1.5ml) containing 500 µL of TSB agar and incubated at 37°C for 72 hours. The resulting colonies were frozen at -20°C overnight, followed by one hour at -80°C. After the cultures were thawed at room temperature, 400 µL of 50% ACN was added to the microfuge tube and placed on a rotary shaker for 30 minutes. The suspension was subjected to centrifugation at 3000×*g* for 5 minutes. Direct injection ESMS analyses were then performed on a Waters Synapt G2 quadrupole TOF mass spectrometer (Milford, MA, USA). Thereafter, 3 µL of the culture supernatants were injected into the ESMS and subjected to an electrospray ionization source with a cone voltage of 15V and capillary voltage of 2.5 kV. The desolvation temperature was set to 275°C and nitrogen was used as desolvation gas at 650 L/hour. Data was collected by scanning over an *m/z* range of 300 to 2000 in the positive mode. The resulting spectra were then analysed with MassLynx V4.1 software (Waters Inc., Milford, MA, USA). Isolate ESMS spectra were compared to that of the negative control (sterile TSB medium extracted similar to isolates) and positive control (*Br. parabrevis*). ESMS spectra were charge deconvoluted with the MaxEnt algorithm, included in the MassLynx V4.1 software, to obtain experimental monoisotopic M_r values where specified. ESMS experiments were done at the LC-MS Central Analytical Services Unit, Science Faculty, Stellenbosch University, South Africa.

2.3.6 Bacterial identification

MALDI-MS biotyping was done using identical sample preparation methodology and machinery as described in section 2.3.4, with the aim to rapidly identify the producer bacterial species. The linear positive mode was used to acquire spectra within a *m/z* range of 3000 to 15000. The initial laser power was set to 50% with a maximal laser power of 80% to obtain peaks. FlexControl software version 3.4 (Bruker Daltonics, Bremen, Germany) in autoexecute mode was used to automatically acquire spectra by rastering the target spot with

the default pattern, including a 10 times spectrum collection with a laser frequency of 1000 Hz. The software package MALDI Biotyper Real Time Classification (Bruker Daltonics, Bremen, Germany) was used to automatically process the peptide spectra of the accumulated 3,000 shots. The software performs steps of smoothing and baseline correction during peak evaluation. The peak resolution was set to be greater than 400 for the identification of the most significant peaks. Spectra acquired from each sample were compared to a reference database containing 4110 microorganisms. MALDI-MS biotyping was done at the Proteomics Research & Services Unit, Department of Biotechnology, University of the Western Cape, South Africa.

Bacterial identification using 16S rRNA gene sequencing was done by the laboratory of Prof. K. Jacobs at the Department of Microbiology, Stellenbosch University, South Africa. 16S rRNA gene sequencing provides a more accurate identification than MALDI-MS biotyping methodology and was therefore done to confirm the identity of the producer bacterial species. Bacterial colonies were inoculated in 1 mL NB broth and incubated at 30°C overnight. DNA was then extracted using the ZR Fungal/bacterial DNA kit and the presence of genomic DNA was checked on a 1% agarose gel, stained with ethidium bromide. Polymerase chain reactions (PCR) were done using a GeneAmp PCR System 9700 (AppliedBiosystems, USA). The reaction mixture contained 0.5 µL (±50 ng/µL) of the purified genomic DNA, 500 nM of each primer and 5 µl of 2X KapaTaq Readymix in a total volume of 10 µL. Bacterial specific primers (8F primer 5'-AGAGTTTGATCCTGGCTCAG-'3 and 1492R primer 5'-TTACCTTGTTACGACTT-'3) were used [20]. The PCR conditions consisted of initial denaturing step at 94°C for 10 min, followed by 36 cycles of 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 1 minute. The reaction was completed with a final extension for 7 minutes at 72°C and then cooled and held at 4°C. PCR samples were separated on a 1% agarose gel, stained with ethidium bromide and visualized using ultraviolet light. The

amplicons from the PCR reactions were run on an ABI 3010xl Genetic analyser from Applied Biosystems (California, USA). Sequences were blasted against the NCBI Genbank database.

2.4 Results and discussion

2.4.1 The isolation of antimicrobial producing bacteria

The undisclosed commercial soil additive in this study is routinely used in agriculture as a bacterial soil inoculant to introduce beneficial bacteria into the soil that can potentially produce a more viable and stable environment for plant life. This commercial product contains three main undisclosed bacterial species. Aliquots from two separate soil additive containers were spread onto three different agar media (TSB, LB and NB) to facilitate the growth of both fastidious and non-fastidious bacteria. Due to the low concentration of viable bacterial cells in the soil additive, no serial dilutions were necessary to produce single colonies.

Morphologically different colonies were selected from each agar growth medium which resulted in a total of 33 distinctive isolates. However, it was found that some isolate colonies originating from different growth media displayed similar colony morphologies (Table 2.1). This may be due to differences in the nutrient composition of growth media resulting in morphological variations [21,22]. Colony morphologies of isolates grown on different media can therefore not be compared. Thus, morphologically similar colonies isolated from different nutrient media were included in further analyses.

Following isolation, the colonies were subjected to simultaneous antagonism spot-on-lawn analysis and blood plate analysis to determine their antimicrobial activity towards *M. luteus* and haemolytic activity respectively [18,19]. From the 33 isolates only 18 were active against *M. luteus* with only two isolates, LB.c.2.4 and LB.c.2.5, displaying higher activity compared to *Br. parabrevis* (the positive control) (Table 2.2). Blood plate analysis revealed that 24 of

the selected isolates displayed haemolytic activity, 11 of which had higher haemolytic activity than *Br. parabrevis* (Table 2.3).

Table 2.1 Summary of the different colony morphologies that were identified in the commercial soil additive from three different growth media.

Isolate [a]	Colour	Form	Margin	Elevation	Size	Texture	Appearance
LB.c.1.1[1]	Cream	Circular	Entire	Flat	Moderate	Smooth	Shiny
LB.c.1.2[2]	Cream	Irregular	Lobate	Flat	Large	Rough	Dull
LB.c.1.3[3]	Cream	Circular	Entire	Flat	Small	Smooth	Shiny
LB.c.1.4	Cream	Circular	Curled	Flat	Small	Smooth	Dull
LB.c.1.5	Cream	Irregular	Entire	Flat	Large	Smooth	Dull
LB.c.1.6	Cream	Irregular	Lobate	Flat	Moderate	Rough	Dull
LB.c.2.1	White	Circular	Entire	Flat	Small	Smooth	Shiny
LB.c.2.2[4]	Cream	Circular	Entire	Raised	Small	Smooth	Shiny
LB.c.2.3	Cream	Circular	Undulate	Flat	Moderate	Smooth	Shiny
LB.c.2.4	White	Circular	Entire	Flat	Moderate	Rough	Dull
LB.c.2.5	Cream	Irregular	Undulate	Flat	Moderate	Rough	Dull
NB.c.1.1[5]	Cream	Circular	Entire	Flat	Moderate	Rough	Shiny
NB.c.1.2	Cream	Irregular	Rhizoid	Flat	Moderate	Rough	Dull
NB.c.1.3[6]	Cream	Circular	Entire	Raised	Moderate	Smooth	Shiny
NB.c.1.4[2]	Cream	Irregular	Lobate	Flat	Large	Rough	Dull
NB.c.1.5	Cream	Spindle	Entire	Raised	Moderate	Smooth	Shiny
NB.c.2.1[4]	Cream	Circular	Entire	Raised	Small	Smooth	Shiny
NB.c.2.2[3]	Cream	Circular	Entire	Flat	Small	Smooth	Shiny
NB.c.2.3	Cream	Irregular	Entire	Raised	Moderate	Smooth	Shiny
NB.c.2.4	Cream	Spindle	Lobate	Flat	Moderate	Rough	Dull
NB.c.2.5	Cream	Circular	Entire	Flat	Large	Smooth	Shiny
NB.c.2.6[1]	Cream	Circular	Entire	Flat	Moderate	Smooth	Shiny
TSB.c.1.1	Cream	Irregular	Lobate	Umbonate	Moderate	Rough	Dull
TSB.c.1.2[5]	Cream	Circular	Entire	Flat	Moderate	Rough	Shiny
TSB.c.1.3	White	Irregular	Lobate	Umbonate	Large	Rough	Dull
TSB.c.1.4[6]	Cream	Circular	Entire	Raised	Moderate	Smooth	Shiny
TSB.c.1.5[1]	Cream	Circular	Entire	Flat	Moderate	Smooth	Shiny
TSB.c.1.6	Cream	Irregular	Lobate	Umbonate	Large	Rough	Dull
TSB.c.2.1[3]	Cream	Circular	Entire	Flat	Small	Smooth	Shiny
TSB.c.2.2	Cream	Irregular	Curled	Flat	Moderate	Smooth	Shiny
TSB.c.2.3	Cream	Irregular	Undulate	Flat	Moderate	Smooth	Shiny
TSB.c.2.4	Cream	Irregular	Undulate	Umbonate	Moderate	Smooth	Shiny
TSB.c.2.5	Cream	Irregular	Undulate	Flat	Large	Smooth	Shiny

Traditional bacterial colony morphology characteristics were visually determined from agar growth media.

[a] Isolates were named as follows: growth media abbreviation followed by.c.1 or c.2 (soil inoculant container number).and number of the isolate. Isolates from different media displaying similar colony morphologies are indicated by [1-6].

Table 2.2. Antimicrobial activity of morphologically different isolates towards *M. luteus*

Isolate	Growth [a]	Antimicrobial Activity [b]
LB.c.1.1	#	+
LB.c.1.2	+	-
LB.c.1.3	#	+
LB.c.1.4	#	+
LB.c.1.5	+	-
LB.c.1.6	+	-
LB.c.2.1	#	+
LB.c.2.2	+	-
LB.c.2.3	+	-
LB.c.2.4	#	++
LB.c.2.5	+	++
NB.c.1.1	#	+
NB.c.1.2	+	-
NB.c.1.3	+	+
NB.c.1.4	+	-
NB.c.1.5	#	+
NB.c.2.1	#	+
NB.c.2.2	+	+
NB.c.2.3	+	-
NB.c.2.4	+	-
NB.c.2.5	+	-
NB.c.2.6	+	-
TSB.c.1.1	+	-
TSB.c.1.2	#	+
TSB.c.1.3	+	+
TSB.c.1.4	#	+
TSB.c.1.5	#	+
TSB.c.1.6	+	-
TSB.c.2.1	+	-
TSB.c.2.2	+	+
TSB.c.2.3	+	±
TSB.c.2.4	+	±
TSB.c.2.5	+	-

[a] The colony size (growth) of isolates were compared to that of *Br. parabrevis* after 48 hours. The + sign is indicative of similar colony size whereas # refers to a large swarming colony.

[b] Antimicrobial activity was determined by comparing the size of clear inhibition zones produced by isolates to the inhibition zone formed by *Br. parabrevis* after 48 hours. The + sign is used to indicate inhibition zones of similar size to that formed by *Br. parabrevis*, whereas ± and ++ refers to smaller and larger inhibition zones respectively. Absence of inhibition zone is indicated by -.

Table 2.3. Haemolytic activity of morphologically different isolates

Isolate	Growth [a]	Haemolytic Activity [b]	
		24 h	48 h
LB.c.1.1	+	+	++
LB.c.1.2	+	-	±
LB.c.1.3	+	±	++
LB.c.1.4	+	±	+
LB.c.1.5	+	-	±
LB.c.1.6	+	-	±
LB.c.2.1	+	+	++
LB.c.2.2	+	-	-
LB.c.2.3	+	-	-
LB.c.2.4	+	+	++
LB.c.2.5	+	-	±
NB.c.1.1	+	+	+
NB.c.1.2	+	-	-
NB.c.1.3	+	+	++
NB.c.1.4	+	-	±
NB.c.1.5	+	+	+
NB.c.2.1	+	+	++
NB.c.2.2	+	+	++
NB.c.2.3	+	-	-
NB.c.2.4	+	-	±
NB.c.2.5	+	-	-
NB.c.2.6	+	-	-
TSB.c.1.1	+	-	-
TSB.c.1.2	+	+	++
TSB.c.1.3	+	-	±
TSB.c.1.4	+	+	++
TSB.c.1.5	+	+	++
TSB.c.1.6	+	-	±
TSB.c.2.1	+	-	-
TSB.c.2.2	+	+	++
TSB.c.2.3	+	-	±
TSB.c.2.4	+	-	±
TSB.c.2.5	+	-	-

[a] The colony size (growth) of isolates were compared to that of *Br. parabrevis* after 48 hours of incubation. The + sign is indicative of a colony size similar to that of *Br. parabrevis*.

[b] Clear haemolytic zones sizes were compared to the haemolytic zone size produced by *Br. parabrevis* after 24 and 48 hours. The + sign is used to indicate haemolytic zones of similar size to those formed by *Br. parabrevis*, whereas ++ and ± refers to a larger haemolytic zone and smaller haemolytic zone respectively. The - sign is indicative of the absence of a haemolytic zone.

Comparing morphological characteristics, colony growth, antimicrobial activity and haemolytic activity of the isolates, it was found that the isolate LB.c.1.1 was similar to the isolate TSB.c.1.5 and the isolate LB.c.1.2 was similar to the isolate NB.c.1.4. Thus, approximately 31 distinct bacterial isolates were obtained from the commercial soil additive. This exceeds the number stipulated by the manufacturer, however, the soil additive containers were not handled under aseptic conditions and could therefore have been contaminated with additional bacterial species. Genomic analysis will have to be done to determine the true number of isolates, however, this is outside the scope of this study.

Two of the isolates, namely LB.c.2.4 and LB.c.2.5, were selected for further investigation including antimicrobial peptide production and purification, due to higher antimicrobial activity towards *M. luteus* as compared to other isolates and the positive control, *Br. parabrevis* (Table 2.2). For simplicity these isolates were renamed LB.4 and LB.5. Although the antimicrobial activity of the LB.4 and LB.5 isolates towards *M. luteus* were similar, the LB.4 isolate displayed higher haemolytic activity than the LB.5 isolate (Table 2.3).

The antimicrobial activity toward *M. luteus* and haemolytic activity were confirmed by performing three independent spot-on-lawn experiments in triplicate (Figure 2.1). The LB.4 isolate was shown to form clear inhibition zones comparable to *Br. parabrevis* (Figure 2.1 A). Interestingly, the LB.4 isolate formed smaller, circular colonies during the confirmation experiments, whereas swarming colonies were observed during the initial spot-on-lawn analysis. Swarming is the cooperative translocation of bacterial colonies on a semi-solid surface so as to find a more nutrient rich niche [23,24]. Thus, the formation of swarming colonies during the initial screening might be due to a low media bed height, resulting in decreased nutrient concentration per surface area. Additionally, it has been shown that swarming is linked to the production of extracellular wetting agents so as to decrease the surface tension of the agar and aid in bacterial motility [24]. Julkowska *et al.* [25]

demonstrated the necessity of the biosurfactant, surfactin, in the swarming of *Bacillus subtilis*. Swarming might, therefore, be seen as a positive quality as many biosurfactants, including surfactin, have been shown to possess potent antimicrobial activity [26].

During confirmation experiments, the LB.5 isolate displayed larger inhibition zones than both *Br. parabrevis* and the LB.4 isolate, however, the zones were not fully translucent (Figure 2.1 A). This might be indicative of late production of the antimicrobial agents only after an established colony has formed. Consequently, *M. luteus* will grow and establish in the deeper part of the agar while the LB.5 isolate is not yet producing any antimicrobials. In contrast, this might also indicate that the antimicrobial agent(s) produced by the LB.5 isolate cannot diffuse deep into the agar due to chemical properties such as hydrophobicity, size and charge.

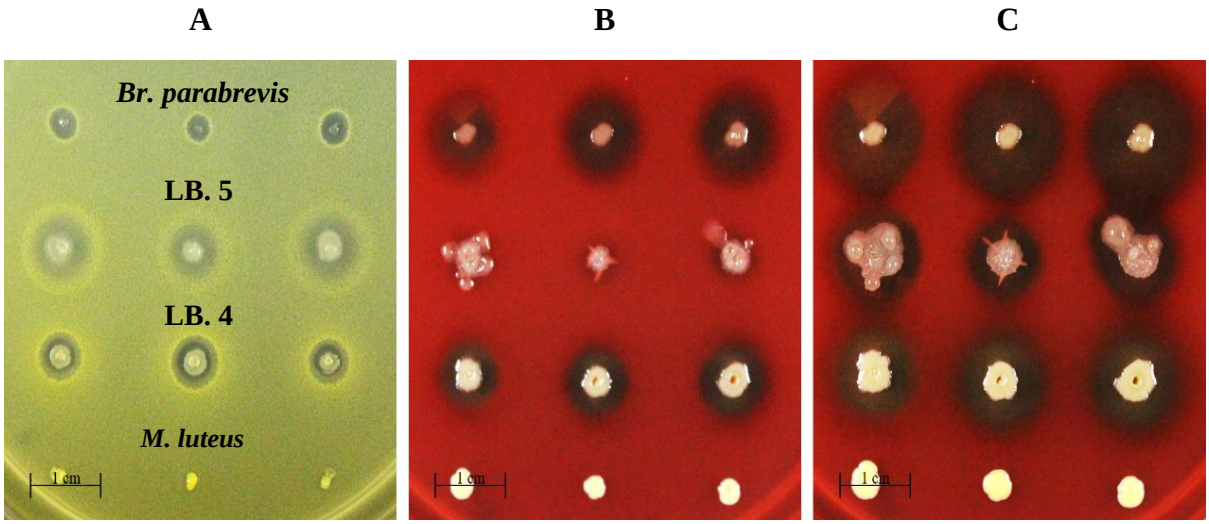


Figure 2.1. Antimicrobial activity of the LB.4 and LB.5 isolates towards *M. luteus* after 48 hours (A). Haemolytic activity of the LB.4 and LB.5 isolates at 24 hours (B) and 48 hours (C). Three biological repeats were performed in triplicate.

Haemolytic spot plate analyses revealed that the LB.4 isolate produces a haemolytic agent after 24 hours and that its production increases over time up until 48 hours (Figure 2.1 B and C). However, the LB.5 isolate only started to display haemolytic activity after 48 hours (Figure 2.1 B and C), which may support the above hypothesis that the LB.5 isolate only starts producing its antimicrobial agent(s) after the colony has been well established. These

results demonstrate that both the isolates, LB.4 and LB.5, are possible candidates for the production of antimicrobial compound(s) and possibly peptides.

2.4.2 MALDI-MS and ESMS analysis of low M_r compounds produced by the LB.4 and LB.5 isolates

After 72 hours of incubation, the LB.4 and LB.5 isolates were subjected to MALDI-MS analysis from *m/z* 500 to 3000 to determine their respective low M_r compound profiles. *Br. parabrevis* was used as a positive control because it produces the previously characterised anticmicrobial peptide group collectively referred to as tyrocidines. The M_r values obtained for the major tyrocidine analogues namely tyrocidine A, tyrocidine B, tyrocidine C, tryptocidine A, tryptocidine B and tryptocidine C were compared to their theoretical M_r values to determine the validity and accuracy of the methods. Theoretical monoisotopic M_r values were calculated from tyrocidine analogue amino acid sequences, previously determined by Tang *et al*. [27], as the sum of the constitutive amino acids' M_r values [28]. The UltrafleXtreme MALDI TOF/TOF mass spectrometer was calibrated for high M_r molecules in the 3-15 kDa range for bacterial strain identification, which led to an average positive M_r error of 3.79 in the observed low M_r values between 500 to 2000 (Table 2.4 and Figure 2.2). The M_r values of the peptides determined with MALDI-MS were therefore not accurate but were corrected by subtracting 3.79 atomic mass units.

Problems encountered with lower M_r range calibration of the UltrafleXtreme MALDI TOF/TOF mass spectrometer, as well as the cost of peptide profile analysis led us to develop a rapid ESMS method for the analysis of low M_r compounds produced by bacterial colonies. Bacterial colonies to be identified were spot onto TSB agar media in microfuge tubes (1.5 mL). The cultures were subsequently incubated for 72 hours after which they are frozen at -20°C followed by a short freezing step at -80°C to ensure the samples are frozen throughout. Since it is not known whether the antimicrobial compounds are intracellular and/or

extracellular, the initial slow freeze step facilitates cell lysis. Cell lysis ensures the release of intracellular antimicrobial compounds. After thawing at room temperature, the cells and TSB agar media were directly extracted with 50% ACN and subjected to ESMS analysis from *m/z* 300 to 2000 to determine the intra- and extracellular low M_r profiles collectively. The ACN extraction was an improvement on the MALDI-MS analysis, which will preferentially identify only high amounts of small extracellular compounds due to the interference of the intracellular matrix.

Major low M_r compounds observed with MALDI-MS and ESMS of *Br. parabrevis*, correlated with the M_r of known major tyrocidine analogous (Figure 2.2 and Table 2.4) [27,28]. Tyrocidine A, tyrocidine B, tyrocidine C, tryptocidine A, tryptocidine B, tryptocidine C were detected by the ESMS method, whereas MALDI-MS only detected tyrocidine A, tyrocidine B and tyrocidine C (Figure 2.2 A and C). The tryptocidines are produced at lower concentrations than the tyrocidines. This result confirmed the higher sensitivity and overall accuracy at lower M_r values of our ESMS method. Furthermore, the ESMS spectra of the direct extracts also revealed the presence of expected gramicidins (Figure 2.2 B) and oligomers (data not shown) of the various tyrocidines [27]. The analysis of the small direct extracts of the tyrocidine producer demonstrated the validity of the ESMS method for rapid, sensitive detection and profiling of the low M_r compounds produced by bacterial colonies from solid nutrient growth media. Furthermore, this method has an advantage when compared to MALDI-MS in that electrospray tandem mass spectrometry (ESMS/MS) analysis, as well as ultra performance liquid chromatography (UPLC) linked to ESMS can be performed directly and therefore fast tracking the identification of discovered antimicrobials.

MALDI-MS of the major compounds produced by the LB.4 isolate indicated compounds with corrected M_r values at: 1272.609, 1519.317, 1584.298 and 1623.244 (Figure 2.3 A).

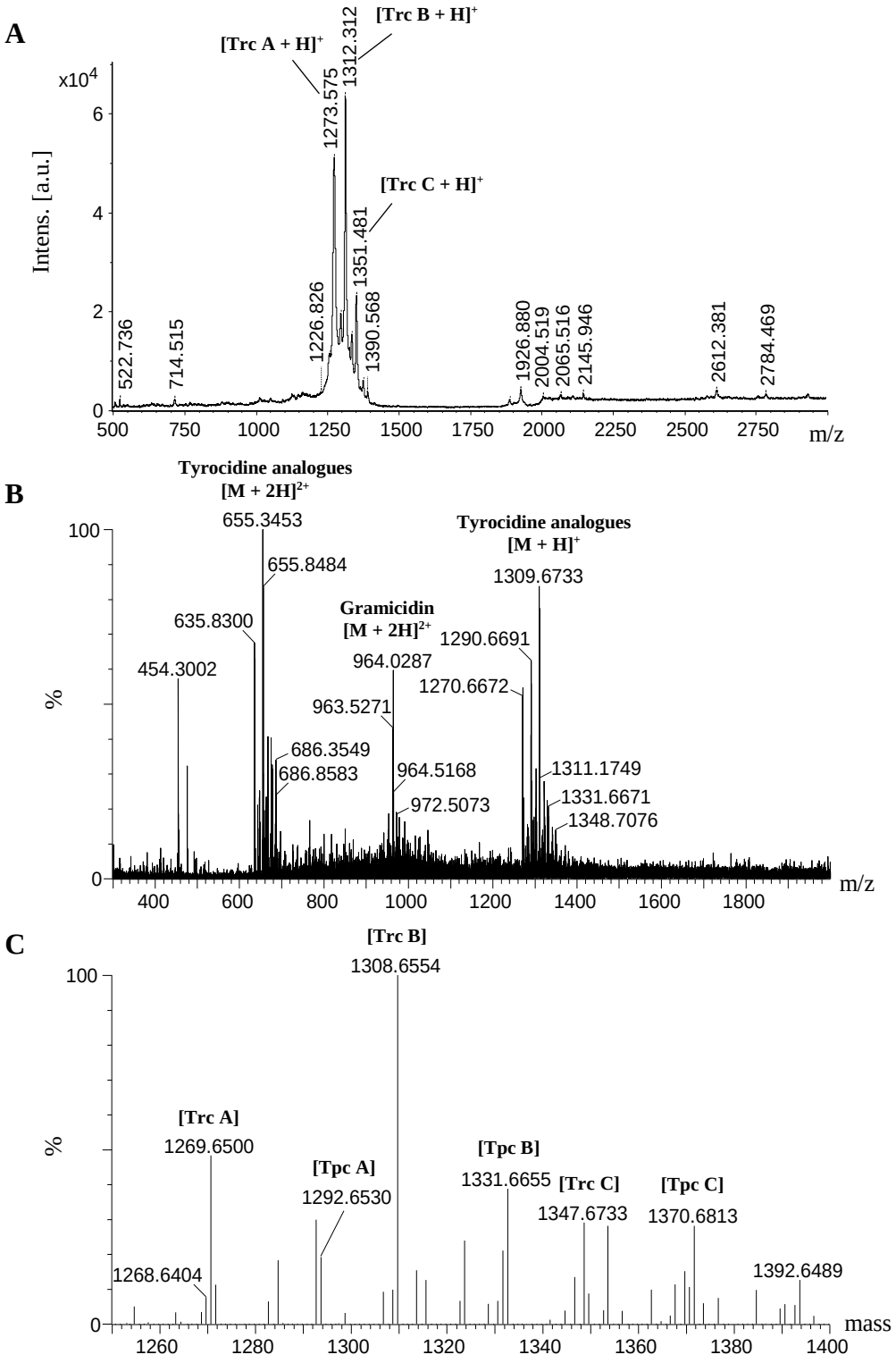


Figure 2.2. MALDI-MS and ESMS analysis of the low M_r compounds produced by *Br. parabrevis*. A: MALDI-MS spectrum from m/z 500-3000 of *Br. parabrevis* displaying tyrocidine analogues; B: The ESMS spectrum from m/z 300-2000 of *Br. parabrevis* show the singly ($[M + H]^+$) and doubly ($[M + 2H]^{2+}$) charged species of tyrocidine analogues as well as the doubly charged ($[M + 2H]^{2+}$) gramicidin species; C: MaxEnt analysis of the ESMS spectrum in B show the M_r values between 1250 and 1400 of the tyrocidine analogues. Tyrocidine and tryptocidine are abbreviated as Trc and Tpc respectively. Note, the MALDI-MS spectrum (A) does not display accurate *m/z* values due to calibration errors.

Table 2.4. Summary of M_r values of the major tyrocidine analogues produced by *Br. parabrevis* as determined by ESMS and MALDI-MS.

Major tyrocidine analogues	Abbr.	Sequence [a]	Theoretical monoisotopic M_r [b]	ESMS monoisotopic M_r [c]	MALDI-MS M_r	MALDI-MS corrected M_r [d]
tyrocidine A	Trc A	*Cyclo*-(VOLfPFfNQY)	1269.6546	1269.6500	1273.575	1269.785
tyrocidine B	Trc B	*Cyclo*-(VOLfPWfNQY)	1308.6655	1308.6554	1312.312	1308.522
tyrocidine C	Trc C	*Cyclo*-(VOLfPWwNQY)	1347.6764	1347.6733	1351.481	1347.691
tryptocidine A	Tpc A	*Cyclo*-(VOLfPFfNQW)	1292.6706	1292.6530	-	-
tryptocidine B	Tpc B	*Cyclo*-(VOLfPWfNQW)	1331.6815	1331.6655	-	-
tryptocidine C	Tpc C	*Cyclo*-(VOLfPWwNQW)	1370.6924	1370.6813	-	-

M_r values were obtained from ESMS spectra (Figure 2.2 C) and MALDI-MS spectra (Figure 2.2 A) respectively

[a] Amino acid sequences as determined by Tang *et al*. [27].Conventional one-letter abbreviations for amino acids were used, except for ornithine which is depicted by "O". D-amino acid residues are illustrated as lower case conventional one-letter abbreviations.

[b] Theoretical monoisotopic M_r were calculated as the sum of M_r's of constituent amino acids.

[c] Experimental monoisotopic M_r as determined by the MaxEnt algorithm.

[d] Low M_r calibration errors of the UltrafleXtreme MALDI TOF/TOF mass spectrometer resulted in an average M_r increase of 3.79. The original MALDI-MS results were therefore converted by the subtraction of 3.79 atomic mass units.

Figure 2.3 B and C displays the ESMS spectra of low M_r compounds produced by the LB.4 isolate. The M_r values of major compounds produced by the LB.4 isolate include: 881.5872, 895.6042, 1222.6388, 1272.6116, 1569.0597 and 1583.0856.

Literature was reviewed to determine whether the M_r of major compounds obtained by MALDI-MS and ESMS analysis of the LB.4 isolate correlated with previously discovered antimicrobial agents. From the literature it was found that the isolate LB.4 produced two compounds with M_r values of 1569.0597 and 1583.0856 which closely correlated with that of the antimicrobial peptides BT1569 (M_r = 1569.0097) and BT1583 (M_r = 1583.1154) respectively [29]. Wu *et al*. [29] found that BT1569 and BT1583 are non-ribosomally produced, together with several closely related peptides collectively referred to as BT peptides, by *Brevibacillus texasporus* via the BT non-ribosomal peptide synthetase operon (BT NRPS) [29]. Furthermore, they determined the amino acid sequences of BT1569 and BT1583, as well as their antimicrobial activity towards *Staphylococcus aureus* [29]. No further characterisation studies, however, have been published on these peptides and therefore several key areas still need to be investigated. This includes antimicrobial activity against various pathogens, mode of action, three dimensional structure determination and structure-activity relationships. It should, however, be noted that ESMS analysis was done at high resolution (< 10 ppm mass error). The compounds produced by the LB.4 isolate with M_r values of 1569.0597 and 1583.0856 display a mass error of 50 ppm and 29 ppm when compared to the M_r values of BT1569 (M_r = 1569.0097) and BT1583 (M_r = 1583.1154), respectively. These mass errors are outside the mass error range and also does not point to a single amino acid mutation. This makes it uncertain whether the LB.4 isolate produces BT1569 and BT1583 peptides.

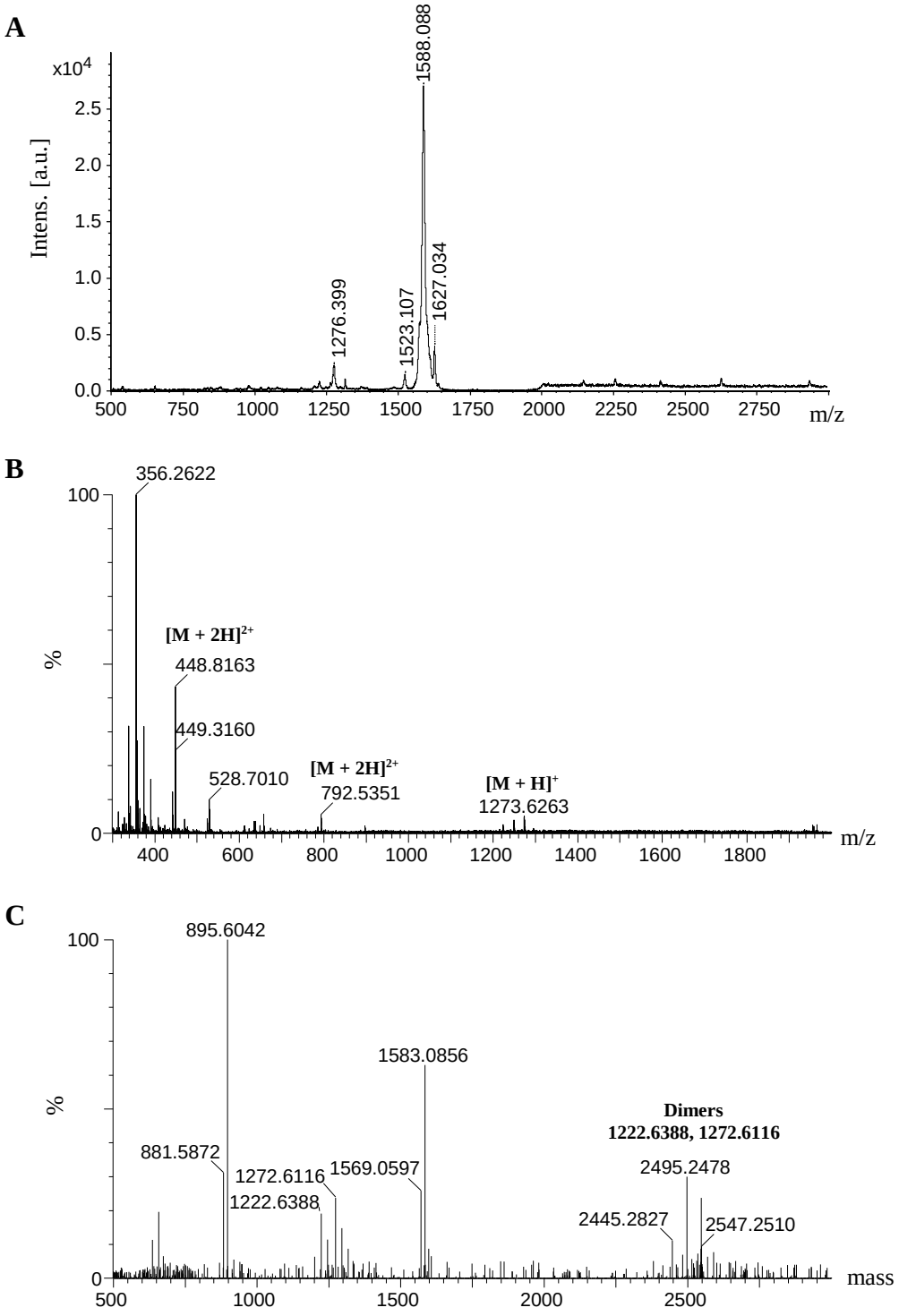


Figure 2.3. MALDI-MS and ESMS analysis of the low M_r compounds produced by the isolate, LB.4. A: MALDI-MS spectrum from *m/z* 500-3000; B: The ESMS spectrum from *m/z* 300-2000 of the LB.4 isolate extracts show singly ($[M + H]^+$) and doubly ($[M + 2H]^{2+}$) charged species; C: MaxEnt analysis of the ESMS spectrum in B displaying M_r values from 500 to 3000. M_r values displayed in A and C are indicative of the major compounds, suspected to have antimicrobial activity.

The M_r values of compounds at 1569.0597 and 1583.0856 were also found to be similar to that of the antimicrobial peptides bogorol B (M_r = 1569.0633) and bogorol A (M_r = 1583.0790), respectively [30]. These peptides form part of the bogorol group of five related antimicrobial peptides (bogorols A-E) produced by *Brevibacillus laterosporus* strain PNG-276, isolated from an unidentified marine tube worm [30]. The structural characteristics and range of antimicrobial activities of the bogorol group of antimicrobial peptides have been extensively studied, however, their mode of action has yet to be determined [30]. Bogorol B amd bogorol A displayed a mass error of 4 ppm and 7 ppm when compared to the M_r values of 1569.0597 and 1583.0856 produced by the LB.4 isolate respectively. This makes the bogorol analogues more likely candidates than the BT peptides for the antimicrobial activity of the LB.4 isolate.

Due to the M_r similarities of the compounds produced by LB.4 to both BT peptides and bogorol analogues, it is likely that the LB.4 isolate produces either BT peptides or bogorol peptides. However, these peptides belong to different peptide groups produced by distinct organisms. Thus, no clear conclusion could be made regarding antimicrobial compound identity. Additionally, the presence of several other low M_r compounds in the mass spectra necessitates further investigation. Further studies focusing on production, organic extraction, purification and identification are required to establish whether the antimicrobially active compound(s) from LB.4 are in fact BT1569 and BT1583, bogorol A and bogorol B, or novel. This study will be reported in Chapter 3.

MALDI-MS identified major compounds produced by the LB.5 isolate with corrected M_r values at: 2149.566, 2467.911, 2507.515, 2622.804 and 2943.439 with a low abundance compound at 1422.716 (Figure 2.4). The ESMS spectrum of the LB.5 isolate displayed one major compound with M_r of 1421.7529 and a sodium adduct at 1443.7382 (Figure 2.4 B and

C). Minor compounds were observed with M_r values of 1092.5544, 1110.5886, 1407.7339 and 2957.2441.

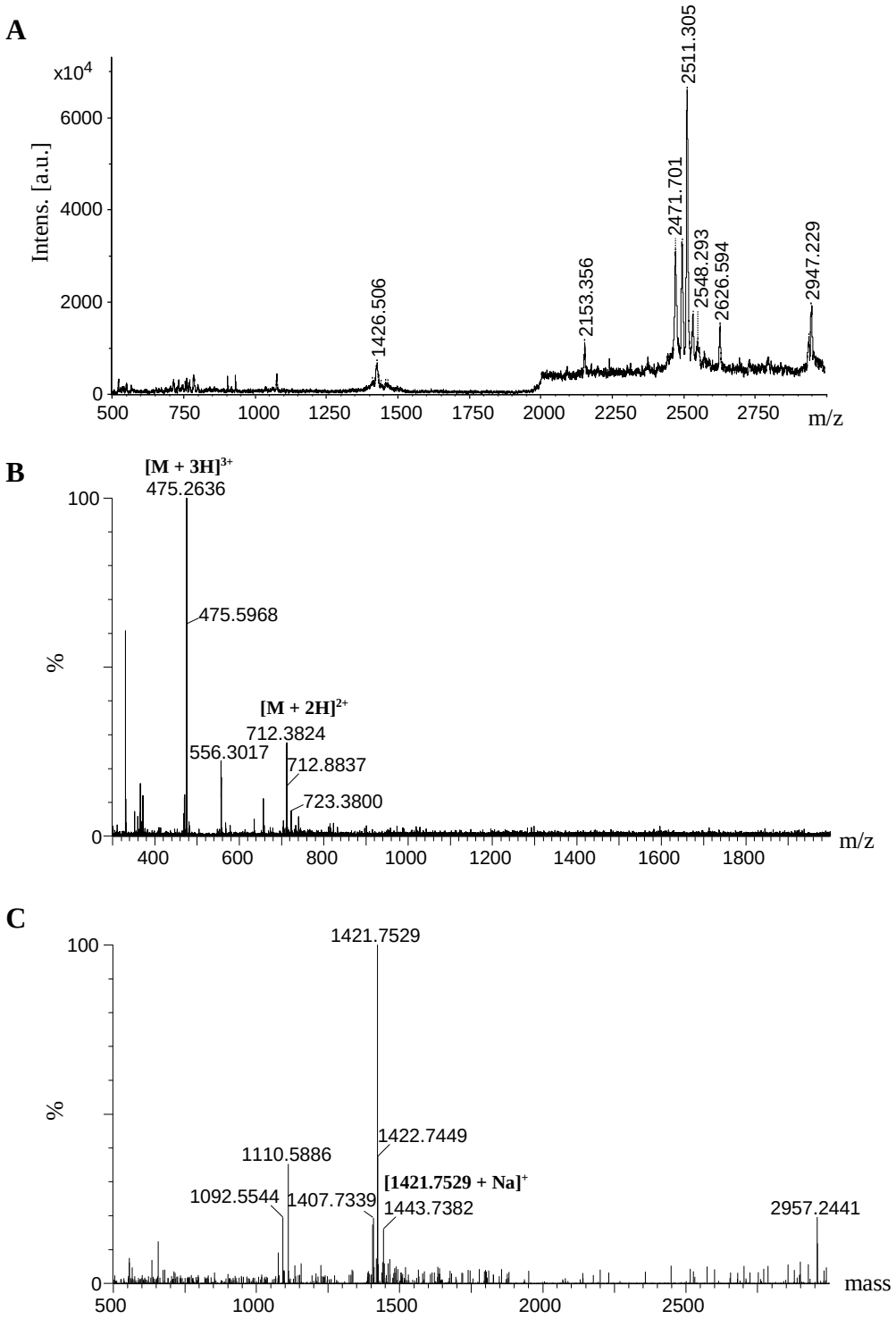


Figure 2.4. MALDI-MS and ESMS analysis of the low M_r compounds produced by the isolate, LB.5. A: MALDI-MS spectrum from *m/z* 500-3000; B: The ESMS spectrum from *m/z* 300-2000 of LB.5 extracts show doubly ($[M + 2H]^{2+}$) and triply ($[M + 3H]^{3+}$) charged species; C: MaxEnt analysis of the ESMS spectrum in B displaying M_r values from 500 to 3000. M_r values displayed in A and C are indicative of the major compounds, suspected to have antimicrobial activity.

A literature review of M_r values of previously discovered antimicrobial agents correlating with the M_r of major and minor compounds produced by the LB.5 isolate, revealed that the M_r of 1421.7529 and 1407.7339 are closely related to that of the well-studied antimicrobial peptides, bacitracin A (M_r = 1421.7489) and bacitracin B (M_r = 1407.7333) respectively [31]. Bacitracin peptides have been studied extensively and are used pharmaceutically, as described in Chapter 1. Its pharmaceutical use partially resulted from the absence of haemolytic activity [32,33]. The low haemolytic activity observed for the LB.5 isolate in section 2.4.1, therefore, further strengthens the possibility of bacitracin production (Table 2.3). Although it is highly probable that the antimicrobial product of the LB.4 isolate could be bacitracin A and bacitracin B, further investigation is needed to confirm this, due to the presence of several low M_r compounds in the mass spectra. The soil additive isolate LB.5 was cultured and extracted to confirm the identity of the antimicrobial compound and to establish whether bacitracin is the sole compound contributing towards the antimicrobial activity of the LB.5 isolate [34]. This study will be reported on in Chapter 3.

2.4.3 Bacterial identification

MALDI-MS biotyping and 16 rRNA gene sequencing were utilised with the aim of identifying the bacterial isolates. Both methods were validated by the correct identification of *Br. parabrevis*, that was used as a positive control (Table 2.5).

The isolate, LB.4, was convincingly identified by MALDI-MS biotyping as *Br. laterosporus* with a biotyping score of 2.164, indicating secure genus identification and probable species identification (Table 2.5). MALDI-MS biotyping identification of the LB.4 isolate was reinforced by 16S rRNA gene sequencing which indicated 99 % nucleotide sequence identity to *Br. laterosporus* (Table 2.5). The strain of *Br. laterosporus* has not yet been determined, therefore, we will refer to the isolate as *Br. laterosporus* LB.4. In section 2.4.2 it was hypothesised that the active antimicrobial compounds produced by *Br. laterosporus* LB.4 are

either BT1569 and BT1583 or bogorol A and B, due to their M_r similarities to compounds detected by MALDI-MS and ESMS. From the bacterial identification it is more likely that the compounds from *Br. laterosporus* LB.4 are bogorol A and bogorol B, as it is known to be produced by a strain of *Br. laterosporus* [30]. However, the BT1583 producer, *Br. texasporus*, is closely related to *Br. laterosporus* [29]. Additionally, *Br. laterosporus* has been shown to produce a variety of structurally diverse antimicrobial peptides such as: tauramamide, BL-A60 and R-1 [35-37]. It is therefore possible that the identities of the antimicrobials produced by *Br. laterosporus* LB.4 have yet to be discovered.

Table 2.5. Summary of bacterial identification as determined by MALDI-MS biotyping and 16S rRNA gene sequencing respectively.

Isolate/Control	MALDI-MS Biotyping		16S rRNA gene sequencing	
	Identification	**Biotyper Score** [a]	**Identification**	**% Sequence identity** [b]
Br. parabrevis ATCC 10068	*Brevibacillus parabrevis*	2.164	*Brevibacillus parabrevis*	99
LB.4	*Brevibacillus laterosporus*	2.259	*Brevibacillus laterosporus*	99
LB.5	*Bacillus sonorensis*	1.857	*Bacillus licheniformis*	99

[a] MALDI-MS biotyping scores reflect as follows: 2.300 - 3 .000 = highly probable species identification, 2.000-2.299 = secure genus identification and probable species identification, 1.700 – 1.999 = probable genus identification, 0.000 – 1.699 = no reliable identification.
[b] Percentage nucleotide sequence identity as determined by NCBI Genbank database blast.

The isolate, LB.5, was identified as *Bacillus sonorensis* by MALDI-MS biotyping with a MALDI Biotyper score of 1.857 (Table 2.5). A MALDI Biotyper score between 1.700 and 1.999 is only indicative of a probable genus identification and the species identification, *sonorensis*, cannot be viewed as accurate. The more accurate method of 16S rRNA gene sequencing identified the LB.5 isolate as *Bacillus licheniformis* with a 99 % nucleotide sequence identity (Table 2.5). This study will therefore refer to the LB.5 isolate as *B. licheniformis* LB.5, as no strain identification has been done thus far. Under section 2.4.2 it was hypothesised that *B. licheniformis* LB.5 produces the antimicrobial peptides bacitracin A

and bacitracin B which is part of a group of related antimicrobial peptides produced by *B. licheniformis* collectively referred to as bacitracin [31,33]. 16S rRNA gene sequencing, thus, reinforced the previous hypothesis.

2.5 Conclusions

Several unique bacteria were isolated from a commercial soil additive. Spot-on-lawn analyses revealed several isolates that display antimicrobial activity towards the Gram-positive bacterium, *M. luteus*. However, only two of the isolates, LB.4 and LB.5, where selected due to their desirable antimicrobial activity. MALDI-MS biotyping and 16S rRNA gene sequencing identified the isolate LB.4 to be a strain of *Br. laterosporus*. 16S rRNA gene sequencing identified the isolate LB.5 to be a strain of *B. licheniformis*. Strain identities, however, could not be determined. The isolates will therefore be referred to as *Br. laterosporus* LB.4 and *B. licheniformis* LB.5 in this dissertation.

Mass spectrometry analysis revealed that *Br. laterosporus* LB.4 and *B. licheniformis* LB.5 produce several low M_r compounds, making these isolates possible producers of antimicrobial peptides. From literature it was found that compounds produced by both *Br. laterosporus* LB.4 and *B. licheniformis* LB.5 had similar M_r values as previously described antimicrobial peptides. Low M_r compounds produced by *Br. laterosporus* LB.4 resembled the M_r values of two members from either the BT peptide group (BT1569 and BT1583) or bogorol group (bogorol A and bogorol B) of antimicrobial peptides. The latter is more likely as *Br. laterosporus* has been shown to be the producing organism of bogorol antimicrobial peptides [30]. Due to M_r similarities, low haemolytic activity and producer organism identity, it is highly probable that *B. licheniformis* LB.5 produces two of the antimicrobial peptides routinely isolated from *B. licheniformis*, collectively termed bacitracin (bacitracin A and bacitracin B) [31,33]. However, several other low M_r compounds, as observed from the

MALDI-MS and ESMS spectra, might also contribute towards the observed antimicrobial activity of both *Br. laterosporus* LB.4 and *B. licheniformis* LB.5. Further isolation, purification and identification of the antimicrobial compounds produced by *Br. laterosporus* LB.4 and *B. licheniformis* LB.5 are therefore necessary to establish identities of the antimicrobial compounds that are produced and whether or not they are novel (Chapter 3). Subsequently, the purified antimicrobials were subjected to activity and cell membrane interaction characterisation as reported on in Chapter 3.

2.6 References

1. Craik, D. J., Fairlie, D. P., Liras, S. and Price, D. (2013) The future of peptide-based drugs. Chem. Biol. Drug Des. **81**, 136-147
2. Epand, R. M. and Vogel, H. J. (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta. **1462**, 11-28
3. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria?. Nat. Rev. Microbiol. **3**, 238-250
4. Nguyen, L. T., Haney, E. F. and Vogel, H. J. (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. **29**, 464-472
5. Garinot-Schneider, C., Pommer, A. J., Moore, G. R., Kleanthous, C. and James, R. (1996) Identification of putative active-site residues in the DNase domain of colicin E9 by random mutagenesis. J. Mol. Biol. **260**, 731-742
6. Stone, K. J. and Strominger, J. L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. U. S. A. **68**, 3223-3227
7. Peschel, A. and Sahl, H. G. (2006) The co-evolution of host cationic antimicrobial peptides and microbial resistance. Nat. Rev. Microbiol. **4**, 529-536
8. Wiedemann, I., Breukink, E., van Kraaij, C., Kuipers, O. P., Bierbaum, G., de Kruijff, B. and Sahl, H. G. (2001) Specific binding of nisin to the peptidoglycan precursor lipid II combines pore formation and inhibition of cell wall biosynthesis for potent antibiotic activity. J. Biol. Chem. **276**, 1772-1779
9. Breukink, E., Wiedemann, I., van Kraaij, C., Kuipers, O. P., Sahl, H. G. and de Kruijff, B. (1999) Use of the cell wall precursor lipid II by a pore-forming peptide antibiotic. Science. **286**, 2361-2364
10. Sang, Y. and Blecha, F. (2008) Antimicrobial peptides and bacteriocins: alternatives to traditional antibiotics. Anim. Health Res. Rev. **9**, 227-235
11. Schwarzer, D., Finking, R. and Marahiel, M. A. (2003) Nonribosomal peptides: from genes to products. Nat. Prod. Rep. **20**, 275-287

12. Bulet, P. and Stocklin, R. (2005) Insect antimicrobial peptides: structures, properties and gene regulation. Protein Pept. Lett. **12**, 3-11

13. Nawrot, R., Barylski, J., Nowicki, G., Broniarczyk, J., Buchwald, W. and Goździcka-Józefiak, A. (2014) Plant antimicrobial peptides. Folia Microbiol. **59**, 181-196

14. Zasloff, M. (2002) Antimicrobial peptides of multicellular organisms. Nature. **415**, 389-395

15. Gould, K. (2016) Antibiotics: from prehistory to the present day. J. Antimicrob. Chemother. **71**, 572-575

16. Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. **25**, 39-67

17. Baltz, R. H. (2008) Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. **8**, 557-563

18. Tagg, J. R., Read, R. S. D. and McGiven, A. R. (1973) Bacteriocin of a group A S*treptococcus*: partial purification and properties. Antimicrob. Agents Chemother. **4**, 214-221

19. Du Toit, E. A. and Rautenbach, M. (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J. Microbiol. Methods. **42**, 159-165

20. Turner, S., Pryer, K. M., Miao, V. P. W. and Palmer, J. D. (1999) Investigating deep phylogenetic relationships among cyanobacteria and plastids by small subunit rRNA sequence analysis. J. Eukaryot. Microbiol. **46**, 327-338

21. Sousa, A. M., Machado, I., Nicolau, A. and Pereira, M. O. (2013) Improvements on colony morphology identification towards bacterial profiling. J. Microbiol. Methods. **95**, 327-335

22. Ben-Jacob, E., Shmueli, H., Shochet, O. and Tenenbaum, A. (1992) Adaptive self-organization during growth of bacterial colonies. Phys. A. **187**, 378-424

23. Daniels, R., Vanderleyden, J. and Michiels, J. (2004) Quorum sensing and swarming migration in bacteria. FEMS Microbiol. Rev. **28**, 261-289

24. Déziel, E., Lépine, F., Milot, S. and Villemur, R. (2003) *rhlA* is required for the production of a novel biosurfactant promoting swarming motility in *Pseudomonas aeruginosa*: 3-(3-hydroxyalkanoyloxy) alkanoic acids (HAAs), the precursors of rhamnolipids. Microbiology. **149**, 2005-2013

25. Julkowska, D., Obuchowski, M., Holland, I. B. and Seror, S. J. (2005) Comparative analysis of the development of swarming communities of *Bacillus subtilis* 168 and a natural wild type: critical effects of surfactin and the composition of the medium. J. Bacteriol. **187**, 65-76

26. Seydlová, G. and Svobodová, J. (2008) Review of surfactin chemical properties and the potential biomedical applications. Cent. Eur. J. Med. **3**, 123-133

27. Tang, X., Thibault, P. and Boyd, R. K. (1992) Characterisation of the tyrocidine and gramicidin fractions of the tyrothricin complex from *Bacillus brevis* using liquid chromatography and mass spectrometry. Int. J. Mass Spectrom. Ion Processes. **122**, 153-179

28. Vosloo, J. A., Stander, M. A., Leussa, A. N., Spathelf, B. M. and Rautenbach, M. (2013) Manipulation of the tyrothricin production profile of *Bacillus aneurinolyticus*. Microbiology. **159**, 2200-2211

29. Wu, X., Ballard, J. and Jiang, Y. W. (2005) Structure and biosynthesis of the BT peptide antibiotic from *Brevibacillus texasporus*. Appl. Environ. Microbiol. **71**, 8519-8530

30. Barsby, T., Warabi, K., Sørensen, D., Zimmerman, W. T., Kelly, M. T. and Andersen, R. J. (2006) The bogorol family of antibiotics: template-based structure elucidation and a new approach to positioning enantiomeric pairs of amino acids. J. Org. Chem. **71**, 6031-6037

31. Siegel, M. M., Huang, J., Lin, B., Tsao, R. and Edmonds, C. G. (1994) Structures of bacitracin A and isolated congeners: sequencing of cyclic peptides with blocked linear side chains by electrospray ionization mass spectrometry. Biol. Mass Spectrom. **23**, 186-204

32. Werner, A. H. and Russell, A. D. (1999) Mupirocin, fusidic acid and bacitracin: activity, action and clinical uses of three topical antibiotics. Vet. Dermatol. **10**, 225-240

33. Johnson, B. A., Anker, H. and Meleney, F. L. (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science. **102**, 376-377

34. Toscano, W. A.,Jr and Storm, D. R. (1982) Bacitracin. Pharmacol. Ther. **16**, 199-210

35. Desjardine, K., Pereira, A., Wright, H., Matainaho, T., Kelly, M. and Andersen, R. J. (2007) Tauramamide, a lipopeptide antibiotic produced in culture by *Brevibacillus laterosporus* isolated from a marine habitat: structure elucidation and synthesis. J. Nat. Prod. **70**, 1850-1853

36. Zhao, J., Guo, L., Zeng, H., Yang, X., Yuan, J., Shi, H., Xiong, Y., Chen, M., Han, L. and Qiu, D. (2012) Purification and characterization of a novel antimicrobial peptide from *Brevibacillus laterosporus* strain A60. Peptides. **33**, 206-211

37. Ren, Z. Z., Zheng, Y., Sun, M., Liu, J. Z. and Wang, Y. J. (2007) Purification and properties of an antimicrobial substance from marine *Brevibacillus laterosporus* lh-1. Wei Sheng Wu Xue Bao. **47**, 997-1001

Chapter 3
Purification, identification and characterisation of antimicrobial peptides produced by bacteria isolated from a soil additive

3.1 Introduction

Antimicrobial peptides are naturally produced by organisms throughout all kingdoms of life and are active against various microorganisms including Gram-positive bacteria, Gram-negative bacteria, fungi and protozoan parasites, such as the malarial parasite *Plasmodium falciparum* [1-3]. The modes of action of antimicrobial peptides are diverse, comprising of intracellular target inhibition and a variety of membrane-disruptive activities [4,5]. Intracellular modes of action have been found to include inhibition of cell wall, DNA and protein synthesis amongst various other mechanisms [2,4,5]. Several mechanisms of membrane-mediated mode of action have been hypothesised (barrel-stave, carpet, toroidal pore and aggregate models), which either result in defined pore formation or detergent-like membrane disruption [6-9]. Antimicrobial peptides associate with cellular membrane phospholipids through non-specific electrostatic and hydrophobic interactions [1,2]. These non-specific associations subsequently result in a low potential for resistance development [1]. Target organisms would have to change large portions of their genetic makeup in order to modify phospholipid characteristics to prevent antimicrobial peptide association. The membrane-disruptive mode of action of antimicrobial peptides has, therefore, become of particular interest as it provides them with a broad spectrum of antimicrobial activity and a lower risk of pathogen resistance development.

The discovery of novel antimicrobial agents has recently become a necessity as many pathogenic organisms have developed resistance towards conventional antibiotics [10].

Antimicrobial peptides have formed the foundation of the search for novel antimicrobial therapeutics due to their rapid, multi-target mode of action and low potential for resistance development [1,11]. With the aim to identify novel antimicrobial peptides, we examined a commercial soil additive for the presence of antimicrobial producing bacteria. This was due to the manufacturer's claim that the additive decreases the frequency of root deceases in many crops. Our screening methodology yielded two bacterial isolates that displayed desirable antimicrobial activity. The isolates were identified to be *Bacillus licheniformis* LB.5 and *Brevibacillus laterosporus* LB.4, respectively. Using an electrospray mass spectrometry (ESMS) technique, developed during the studies reported in this thesis, we found that both isolates produced low molecular mass (M_r) compounds with M_r values similar to that of previously discovered antimicrobial peptides.

B. licheniformis LB.5 was hypothesised to produce the antimicrobial peptides bacitracin A and B, which are of from a group of related antimicrobial peptides collectively referred to as bacitracin [12]. This group of antimicrobial peptides has been extensively studied and has been used in topical antibiotics for many years [13]. Bacitracin elicits its antimicrobial activity by inhibiting the translocation of the peptidoglycan intermediate, *N*-acetylmuramylpentapeptide, from the cytosol to the extracellular environment, which ultimately results in cell wall synthesis inhibition [14]. However, a study done by Storm and Strominger [15] demonstrated the potential of bacitracin to disrupt protoplasts at its minimal inhibition concentration (MIC), suggesting that bacitracin may also possess a membranolytic mode of action. No other studies that focused on the membrane interactions of bacitracin were found in literature, thereby compelling further investigation.

Compounds produced by *Br. laterosporus* LB.4 displayed M_r similarities towards the peptides BT1569, BT1583, bogorol A and bogorol B [16,17]. Bogorol A and B belong to a group of closely related antimicrobial peptides produced by the marine bacterium *Br.*

laterosporus PNG-276 [17]. The BT peptides, on the other hand, are produced by a soil bacterium closely related to *Br. laterosporus,* namely *Br. texasporus* [16]. The antimicrobial and structural properties of these antimicrobial peptides have been reported, however, their modes of action have not yet been determined. Further studies are therefore required to characterise the modes of action of these peptides [16,17].

To illustrate the validity of methods used for the purification, identification and characterisation of antimicrobial peptides from environmental and unknown samples, the antimicrobial peptide tryptocidine C was included in this study. Tryptocidine C is one of several antimicrobial peptide analogues produced by the soil bacterium *Brevibacillus parabrevis*, as part of an antimicrobial peptide complex termed tyrothricin [18]. It has been shown to possess antimicrobial activity towards several Gram-positive bacteria, fungi and *P. falciparum* [3,19-22]. Although we have characterised tryptocidine C, its mode of action has not yet been thoroughly elucidated [3]. It has been suggested that tryptocidine C may elicit its antimicrobial activity in a similar way to other tyrocidines by disrupting the cellular membrane of target organisms, however this exact mechanism has yet to be fully elucidated [3,23,24].

In this study we focused on the purification and identification of the antimicrobial compounds produced by *B. licheniformis* LB.5 and *Br. laterosporus* LB.4. Furthermore, the antimicrobial peptide, tryptocidine C, was purified from *Br. parabrevis* cultures. The purified antimicrobials were subsequently subjected to antimicrobial and haemolytic activity analysis as well as biophysical characterisation. Biophysical characterisation was conducted using ^{31}P solid state nuclear magnetic resonance (NMR) spectroscopy and electrophysiological analysis to elucidate the mode of action of tryptocidine C and the antimicrobial compound(s) produced *B. licheniformis* LB.5. The antimicrobial active compound(s) produced by *Br. laterosporus* LB.4 were included in the antimicrobial and haemolytic activity analysis.

However, they could not be purified in sufficient quantities to be included in biophysical characterisation studies.

3.2 Materials

3.2.1 Bacterial Strains

Cultures of *Br. parabrevis* ATCC 10068, used for the production of tryptocidine C, were supplied by the American Type Culture Collection (Manassas, VA, USA). The National Collection of Type Cultures (Proton Down, Salisbury, United Kingdom) supplied *Micrococcus luteus* NCTC 8340, which was used as an antimicrobial indicator organism. Both *Br. laterosporus* LB.4 and *B. licheniformis* LB.5 were previously isolated in Chapter 2 from a commercial soil additive.

3.2.2 Research materials

Blood from anonymous A+ donors (300 mL enriched erythrocyte fraction with 100 mL of saline-adenine-glucose-mannitol red blood cell preservation solution and 63 mL citrate-phosphate-dextrose anticoagulant) were supplied by the Western Cape Blood services (National Health Laboratory, South Africa) conforming to relevant legislation and ethical guidelines. Merck (Darmstadt, Germany) supplied agar, tryptone soy broth (TSB), yeast extract, tryptone, monopotassium phosphate (KH_2PO_4), sodium chloride (NaCl), disodium phosphate (Na_2HPO_4), calcium chloride ($CaCl_2$), ammonium sulphate, glucose, tris(hydroxymethyl)aminomethane (Tris), ethanol (>99.8) and inorganic salts (for the preparation of TGS growth media). SeaKem® LE Agarose was supplied by Lonza (Basel, Switzerland). Albumax II™ was supplied by Life Technologies (Melbourne, Australia). L-tryptophan, RPMI-1640 medium, hydrozyethyl piperazineethanesulfonic acid (HEPES), sodium bicarbonate, potassium chloride (KCl), phosphorus pentoxide, activated carbon, hypoxanthine, gentamicin, diethyl ether, acetone, chloroform, methanol, octane, formic acid,

trifluoroacetic acid (TFA; >98%), gramicidin S, bacitracin A (71.1% HPLC purity), cholesterol, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE), 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoglycerol (POPG) were purchased from Sigma-Aldrich (St. Louis, USA). Acetonitrile (ACN) (HPLC-grade, far UV cut-off) was from Romill Ltd. (Cambridge, United Kingdom). Petri dishes (90 mm) and 50 mL polypropylene centrifuge tubes were supplied by Lasec (Cape Town, South Africa). Corning Incorporated (New York, USA) supplied flat bottom sterile 96-well microtiter plates. Mixed cellulose syringe filters (0.22 µm) were obtained from Merck-Millipore (Massachusetts, USA). A Nova-Pak HR C18 (6 µm particle size, 60 Å pore size, 7.8 mm x 300 mm) semi-preparative high-performance liquid chromatography (HPLC) column and an Acquity UPLC HSS T3 (2.1 × 1500 mm; 1.8 µm particle size) ultra-performance liquid chromatography column (UPLC) were from Waters (Milford, USA). Micro electrode cavity array (MECA) chips were from Nanion Technologies (Munich, Germany). Analytical grade water was prepared by filtering water from a reverse osmosis plant through a Millipore-Q® water purification system (Milford, USA).

3.3 Methods

3.3.1 Production, isolation and purification of tryptocidine C

The tyrocidine producing bacterium, *Br. parabrevis*, was streaked onto TSB agar (30 g/L TSB, 1.5% agar) plates from freezer stocks. After incubation at 37°C for 48 hours, TSB broth (30 g/L TSB) was inoculated with a single colony from the TSB agar plate and incubated overnight at 37°C while shaking. The resulting overnight culture was sub-cultured into TGS broth (tryptone, glucose and inorganic salts) prepared as described by Lewis *et al*. [25] and incubated at 37°C while shaking until an optical density (OD) of 0.6 (mid logarithmic growth phase) at 620 nm was reached. *Br. parabrevis* produces a large variety of tyrocidine analogues, however, Vosloo *et al.* [26] demonstrated that supplementation of TGS broth

media with tryptophan would shift the tyrocidine analogue profile towards increased tryptocidine C production. Thus, the sub-culture was inoculated into a Erlenmeyer flask containing sterile TGS broth supplemented with 10 mM tryptophan and incubated at 37°C for 10 days. Future commercialisation of this part of the project confines the revealing of most details regarding the production and purification methodologies used in tryptocidine C isolation. The BIOPEP® Peptide Group has determined ideal parameters and media composition for the optimized production of tyrocidine analogues in fermentation vesicles. These conditions are protected under non-disclosure agreements with all BIOPEP® Peptide Group members working on the fermentations to ensure future commercialisation potential of peptide products. Briefly, the extraction process entails the collection of biomass which is then subjected to extraction by extreme pH treatment and organic solvent extraction. Subsequent precipitation steps and activated carbon treatments are utilised for preliminary purification. This yielded a crude extract containing about > 75% tyrothricin. The crude tryptocidine C rich tyrothricin extract was washed with diethyl ether:acetone (1:1 *v/v*) to remove some of the gramicidins and centrifuged for 5 minutes at 3000×*g*. The pellet was collected and dried under nitrogen gas after which it was dissolved in 50% ACN and lyophilised.

Further purification of tryptocidine C from the crude extract was achieved by reverse phase high performance liquid chromatography (RP-HPLC). The chromatography system consisted of a Waters 717 Plus autosampler, Waters 6000A pump, Waters 510 pump and a Waters 440 absorbance detector (set at 254 nm) controlled by Millenium^{32} software (Waters, Milford, USA). The extract was dissolved in 50% ACN at 4 mg/mL and 100 µL injected onto a Nova-Pak HR C18 (6 µm particle size, 60 Å pore size, 7.8 mm x 300 mm) semi-preparative HPLC column. Elution was achieved at a flow rate of 3 mL/min and column temperature set to 35°C with a gradient of decreasing polarity as indicated in Table 3.1; where eluant A and eluant B

consisted 0.1% TFA in water and 10% eluant A in ACN respectively. With the aim to purify tryptocidine C, an ISCO Foxy Jr fraction collector (Lincoln, USA) was set to collect 10 second fractions between the elution time of 9 and 14 minutes which flanked the major peak detected at 254 nm. Subsequently, contribution of tryptocidine C towards the total ESMS signal intensity of all the collected fractions was determined via ESMS using direct injection as described under section 3.3.5. Fractions that contained the highest contribution of tryptocidine C towards the total ESMS signal intensity were pooled after which the purity of the pooled sample, concentrated in 200 µg/mL 3% ACN, was determined by ultra-performance liquid chromatography (UPLC) linked ESMS (ESMS), as described under section 3.3.5.

Table 3.1. RP-HPLC gradient program used to purify tryptocidine C

Time (min)	% Eluant A (0.1% TFA in water)	% Eluant B (10% A in ACN)	Curve type
0.0	50	50	-
0.5	50	50	6 (linear)
23.0	20	80	5 (curve)
24.0	0	100	6 (linear)
26.0	0	100	6 (linear)
30.0	50	50	6 (linear)
35.0	50	50	6 (linear)

3.3.2 Production, isolation and purification of the antimicrobial compound(s) produced by *B. licheniformis* LB.5

Pure colonies of the isolated bacterium *B. licheniformis* LB.5 were obtained from LB agar (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl, 1.5% agar) plates prepared from a freezer stock and incubated for 48 hours at 37°C. Subsequently, LB broth (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) was inoculated with a single colony and incubated overnight at 37°C while shaking. The resulting culture was sub-cultured into sterile LB broth (40x dilution) and incubated on a rotary shaker at 37°C until an OD of 0.6 at 620 nm was reached.

Fresh LB broth was then inoculated with 1% sub-culture and incubated at 37°C for 72 hours while shaking. The cell pellet was removed by centrifuging for 20 min at 15 000×*g*. The resulting supernatant was precipitated with 80% ammonium sulphate overnight at 4°C, after which the precipitate was collected by centrifugation at 15 000×*g* for 20 min and lyophilised.

The resulting crude extract was subjected to spot-on-lawn analysis, as described under section 3.3.6, to confirm the presence of antimicrobial activity towards *M. luteus*. After confirmation of antimicrobial activity, the crude extract was filtered through a 0.22 µm syringe filter to remove insoluble debris. Thereafter the filtrate was again subjected to spot-on-lawn analysis (section 3.3.6) to confirm that the antimicrobial compound was not removed during filtration.

RP-HPLC was utilised to analyse and purify the compound responsible for the antimicrobial properties of the extract. The analysis and purification was done with the chromatographic system described under section 3.3.1. The lyophilised filtrate was dissolved in 50% ACN at a concentration of 20 mg/mL and 100 µL was injected onto the column as described under section 3.3.1. The same eluents as described under section 3.3.1 were used to create the gradient profile depicted in Table 3.2 at a flow rate of 3 mL/min. Fractions were collected from before the injection peak over the full range of peaks detected at 254 nm (2 to 16.5 minutes). After lyophilisation the fractions were dissolved in 400 µL of 50% ACN and then screened for antimicrobial activity using the spot-on-lawn method (section 3.3.6). Fractions that indicated antimicrobial activity were analysed with ESMS, as described under section 3.3.5, to determine the M_r of the active compound(s) as well as its contribution towards the total ESMS signal intensity. Fractions with the highest total ESMS signal intensity contribution of the active compound(s) were pooled and lyophilised. The pooled sample was analytically weighed (section 3.3.4) and dissolved in 3% ACN at 200 µg/mL before the final purity was determined using UPLC-ESMS as described under section 3.3.5.

Table 3.2. RP-HPLC gradient program used to purify antimicrobial compounds from isolate extracts

Time (min)	% Eluant A (0.1% TFA in water)	% Eluant B (10% A in ACN)	Curve type
0.0	90	10	-
0.5	90	10	6 (linear)
13.0	0	100	5 (curve)
14.0	0	100	6 (linear)
20.0	90	10	6 (linear)
25.0	90	10	6 (linear)

3.3.3 Production, isolation and purification of the antimicrobial compound(s) produced by *Br. laterosporus* LB.4

Br. laterosporus LB.4 was streaked onto TSB agar plates from a freezer stock and incubated for 48 hours at 37°C to obtain pure colonies. TSB broth was subsequently inoculated with a single colony and was incubated overnight at 37°C while shaking. The overnight culture was then sub-cultured into sterile TSB broth (40x dilution) and incubated at 37°C shaking. After an OD of 0.6 at 620 nm was reached, sterile TSB broth was inoculated with 1% sub-culture and incubated for 72 hours at 37°C while shaking. Following incubation, the cell pellet and supernatant of the culture was separated by centrifugation at 15000×*g* for 20 minutes. To facilitate cell lysis, the biomass pellet was slowly frozen overnight at -20°C after which the pellet was extracted with 50% ACN and the remaining cell debris removed by centrifugation at 15000×*g* for 20 minutes followed by lyophilisation. The dried extract was dissolved in 50% ACN and insoluble debris again removed with centrifugation at 15000×*g* for 20 minute followed by lyophilisation. The supernatant fraction was subjected to precipitation by 80% ammonium sulphate overnight at 4°C and the supernatant removed by centrifugation at 15000×*g* for 20 minutes. The pellet was dissolved in 50% ACN which resulted in two phases that were allowed to separate under gravity and lyophilised separately.

The three extracts were then subjected to analysis by the spot-on-lawn method (section 3.3.6) and ESMS (section 3.3.5) to screen for antimicrobial activity and M_r similarities respectively. Subsequently, both the pellet and top phase of the supernatant extracts where determined to have similar low M_r profiles and antimicrobial activity. However, the spot-on-lawn analysis indicated higher concentration of the antimicrobial agent(s) in the pellet extract. Therefore, only the extract from the *Br. laterosporus* LB.4 cell pellet was purified by RP-HPLC. The protocol used for purification was the same as described in section 3.3.2, however, the fractions were collected between 1.5 and 16 minutes. The collected fractions were dissolved in 400 µL of 50% ACN and subsequently analysed using the spot-on-lawn method (section 3.3.6) to determine which fractions contained antimicrobial compound(s). Antimicrobial active fractions were analysed with ESMS (section 3.3.5) to determine the M_r and contribution towards the total ESMS signal intensity of the active compound in each fraction. Fractions containing a single antimicrobially active compound with the highest total ESMS signal intensity contribution were pooled and lyophilised. The pooled samples were analytically weighed (section 3.3.4) after which they were concentrated to 200 µg/mL in 3% ACN and analysed with UPLC-ESMS, as described in section 3.3.5, to determine final purity.

3.3.4 Analytical weighing

For analysis the purified peptides, as well as gramicidin S that was used as experimental control, were analytically weighed and aliquoted before being made up into stock solutions. First glass vials were labelled with a diamond tip pen after which they were placed at 120°C for one hour to dry. The vials were cooled under a vacuum for 30 minutes before being placed on a six digit Mettler Toledo XP26 analytical scale (Columbus, USA) which was kept dry by phosphorus pentoxide. A stabilisation time of 12 minutes was allowed before the weight of the vial was recorded every minute for 3 minutes to the nearest microgram. The process of drying followed by weighing was repeated three times and the average used as the

weight of each vial. The peptides were dissolved in 50% ACN and transferred to the analytically weighed vials. After lyophilisation the peptide containing vials were placed on the scale for 12 minutes to allow for stabilisation. Following stabilisation, the weight of the vials were recorded every minute for 3 minutes. The vials were placed back into the lyophiliser for one hour to remove any moisture after which the weight of the vials was again recorded as above. The latter was repeated twice. The average of the measurements was subtracted by the analytical weight of the vial to provide the analytical weight of the peptides. Aliquots were made by dissolving the analytically weighed peptides in 50% ACN to an exact concentration before transferring a known amount to clean vials. The aliquots were subsequently dried by lyophilisation.

3.3.5 Mass spectrometry analysis of extracts and purified antimicrobial compounds

The Waters Synapt G2 quadrupole time-of-flight mass spectrometer connected to a Waters Acquity UPLC and Acquity photo diode array detector (Milford, MA, USA) was used for ESMS, UPLC-ESMS and UPLC linked electrospray tandem mass spectrometry (ESMS/MS) analysis. An electrospray source set with a cone voltage of 15 V, capillary voltage of 2.5 kV and positive mode was used as ionization source. The desolvation temperature was set to 275°C and nitrogen was used as desolvation gas at 650 litre/hour. Crude extracts were dissolved in 50% ACN at a concentration of 1 mg/mL and centrifuged at 8600×*g* for 10 minutes to remove any insoluble particulates before 3 µL was injected and analysed by direct mass analysis.

Fractions collected from RP-HPLC purification, that displayed antimicrobial activity towards *M. luteus*, were dried by lyophilisation after which they were dissolved in 400 µL of 50% ACN and 3 µL was injected for direct mass analysis. Subsequently, the total signal contribution of the major compound in the fractions was determined as a percentage of the

total signal intensities. Fractions with a desirable total signal contribution of the antimicrobial compound(s) were pooled. The pooled samples were analytically weighed before they were dissolved and diluted to 3% ACN at a concentration of 200 µg/mL. Thereafter, 10 µL was injected onto a Waters Acquity UPLC HSS T3 column (2.1 × 1500 mm; 1.8 µm particle size) and separated with a 0.1% (*v/v*) formic acid in water (A) and acetonitrile (B) gradient at a flow rate of 0.4 mL/min. The gradient program was as follows: 100% A from 0 to 0.5 minutes for loading, gradient was from 0 to 58% B from 0.5 to 12 minutes and then 58 to 90% B from 12 to 13 minutes, column wash was at 90% B from 13 to 13.5 minutes, reconditioning was done from 10 to 100% A from 13.5 to 14 minutes and then 100% A from 14 to 17 minutes. Data acquisition for both ESMS and UPLC-ESMS was done by setting the second analyser (MS_2) to scan through the mass/charge ratio (*m/z*) range of 300-2000. The data was then analysed with MassLynx V4.1 software from Waters (Milford, USA). Subsequently the pooled samples were analysed by UPLC-ESMS/MS via collision-induced dissociation. The same column and solvents were used as described above, however, the gradient profile was adapted. The gradient started at 100% A preceding a holding step of 0.5 minutes for sample loading. Gradient was then changed to 30% B over 0.5 minutes. Subsequently, a linear gradient from 30% to 60% B was achieved from 1 to 10 minutes followed by a linear gradient to 80% B from 10 to 15 minutes. The column was then re-equilibrated at 100% A from 15.10 to 18 minutes. A flow rate of 0.3 mL/min was applied throughout the gradient profile. Fragmentation data was generated by ramping the trap collision energy from 20 to 70 V. The fragmentation data was analysed with MassLynx V4.1 software. The MaxEnt algorithm, included in the MassLynx V4.1 software, was used to charge deconvolute ESMS spectra to obtain experimental monoisotopic M_r values where specified.

3.3.6 Spot-on-lawn assay

Antimicrobial activity of extracts and fractions collected from RP-HPLC separation, were determined towards the Gram-positive indicator, *M. luteus*, using an adapted spot-on-lawn method [27,28]. The samples were dissolved in 50% ACN and 10 µL was spot onto LB agar. Extracts were used at a concentration of 10 mg/mL. RP-HPLC fractions, however, were not prepared at a specific concentration due to the small sample quantity. Therefore, RP-HPLC fractions were reconstituted in 400 µL of 50% ACN and spot at an unknown concentration. The positive and negative controls used were 0.5 µg/mL gramicidin S and 50% ACN, respectively. The spots were allowed to dry in a laminar flow hood after which the plates were sterilised by chloroform vapour for 30 minutes. The plates were again placed in the laminar flow hood to remove residual chloroform vapour and then stored at 4°C overnight. Overnight cultures were prepared in LB broth from a single colony of *M. luteus* and incubated at 37°C while shaking. Thereafter, the cultures were sub-cultured in LB broth at 2.5% and incubated until an OD of 0.6 at 620 nm was reached. Molten LB agarose (1% agarose) was inoculated with 10% of the cell suspension and 5 mL poured onto the previously spotted plates. Due to the low melting point of agarose the media could be sufficiently cooled to below 50°C without setting before the cell suspension was added to ensure the viability of *M. luteus*. After 20 hours of incubation at 37°C, photos of the plates were taken with a ChromaDoc-It TLC imaging system (UVP, California, USA) connected to a Canon Eos Rebel T3 digital camera (Canon, Tokyo, Japan). Inhibition zone diameter and area was subsequently determined using ImageJ software V1.49 [29].

3.3.7 Peptide preparation for dose response assays

Stock solutions of the purified peptides were prepared by first dissolving analytically weighed peptides in 100% ethanol. Thereafter they were diluted with analytical grade water to a final concentration of 1.00 mg/mL in 15% ethanol. The stock solutions were two-fold

serially diluted with 15% ethanol from 1.00 mg/mL to 0.980 µg/mL in polypropylene 96-well microtiter plates. The stock solutions and dilution series were freshly prepared 45 minutes before being used in the dose response assays. Dilutions series of the antimicrobial peptide, gramicidin S, were also prepared as described above and used as experimental control during both antimicrobial and haemolytic activity analysis.

3.3.8 Antimicrobial dose response assay

The antimicrobial activity of the peptides towards *M. luteus* were analysed by the micro-broth dilution method as described by Du Toit & Rautenbach [28]. A colony of *M. luteus* selected from LB agar was incubated overnight at 37°C while shaking in LB broth. Thereafter the culture was sub-cultured in TSB growth media at 2.5% and incubated on a rotary shaker at 37°C until an OD of 0.6 at 620 nm was obtained. The cell suspension was diluted with fresh TSB broth to an OD of 0.2 at 620 nm and 90 µL transferred into wells of untreated 96-well microtiter plates. Microtiter plates containing the cell suspension received 10 µL of the peptide dilutions in triplicate resulting in a 100 µL final assay volume, allowing for antimicrobial analysis in the range of 100 µg/mL to 98 ng/mL. Furthermore, ethanol was kept constant at 1.5% which does not affect the growth of *M. luteus*. Background was determined from wells containing only TSB broth with 1.5% ethanol whereas the growth control was determined from wells containing the cell suspension with 1.5% ethanol. After an incubation period of 16 hours at 37°C, the light dispersion of each well was determined spectrophotometrically at 620 nm with a Biotek PowerWave 340 spectrophotometer (Winooski, USA). The percentage growth inhibition was calculated from the light dispersion with equation 4.1 [28]. All assays were conducted with three biological repeats each with triplicate technical repeats.

$$\% \text{ growth inhibition} = \frac{A_{620nm} \text{ of well-(Average } A_{620nm} \text{ of background)}}{\text{Average } A_{620nm} \text{ of total growth - Average } A_{620nm} \text{ of background}} \quad 4.1$$

3.3.9 Haemolytic dose response assay

With the aim to study the toxicity of the peptides towards human cells, haemolytic activity was analysed using a haemolysis assay adapted from Rautenbach *et al*. [22]. Blood was prepared by washing three times with 10.4 g/L RPMI-1640 media supplemented with 4 g/L glucose, 6 g/L HEPES, 2.1 g/L sodium bicarbonate, 5 g/L albumax II, 0.4 g/L hypoxanthine and 50 mg/L gentamicin and removing the supernatant by centrifugation at 1200×*g*. Subsequently, 2% haematocrit in PBS (8 g/L NaCl, 0.2 g/L KCl, 1.44 g/L Na_2HPO_4, 0.24 g/L KH_2PO_4) was transferred to a 96-well microtiter plate at 90 µL/well. The peptide dilutions were added in triplicate (10 µL) to the erythrocytes resulting in a dilution series of 100 µg/mL to 98 ng/mL and a final ethanol concentration of 1.5%. PBS comprising of 2% haematocrit and 1.5% ethanol containing and excluding 100 µg/mL of gramicidin S, represented total and background haemolysis respectively. The plates were incubated at 37°C for two hours. Microtiter plates were subsequently subjected to centrifugation at 200×*g* for 3 minutes to sediment unaffected erythrocytes. Thereafter, 10 µL of the supernatant from each well was transferred to a microtiter plate containing 90 µL PBS in each well. The absorbance at 415 nm of each well was determined spectrophotometrically by a Biotek PowerWave 340 spectrophotometer. The percentage haemolysis was calculated from absorbance readings with equation 4.2 [28].

$$\%\ \text{haemolysis} = \frac{A_{415nm}\ \text{of well} - (\text{Average } A_{415nm}\ \text{of no haemolysis})}{\text{Average } A_{415nm}\ \text{of total haemolysis} - \text{Average } A_{415nm}\ \text{of no haemolysis}} \qquad 4.2$$

3.3.10 Dose response data analysis

For dose response analysis both the growth and haemolytic curves were plotted with GraphPad Prism 4.03 software (San Diego, USA). Non-linear regression was performed and sigmoidal curves with variable slope (Hill slope <7) fitted using equation 4.3. Top and bottom refer to the percentage growth inhibition/haemolysis at high peptide concentration

and in the absence of peptide respectively. The IC_{50}/HC_{50} was calculated from the x-value (log of peptide concentration) from the activity slope (related to the hill coefficient) halfway between the top and bottom plateaus of the sigmoidal curve, representing the peptide concentration needed for 50% growth inhibition/haemolysis. IC_{max} (or calculated MIC) refers to a minimum inhibition concentration calculated from the dose response activity slope intersect for microbial growth inhibition [30].

$$Y = \frac{\text{bottom} + (\text{top} - \text{bottom})}{1 + 10^{IC_{50} \times \text{Activity slope}}} \qquad 4.3$$

3.3.11 Solid state nuclear magnetic resonance spectroscopy

The solid state nuclear magnetic resonance spectroscopy (NMR) section of this study was done in collaboration with the group of Prof B. Bechinger during a research visit to the University of Strasbourg, France. With the aim to elucidate the membrane activity of tryptocidine C and commercial bacitracin A, the ability of the peptides to disrupt macroscopically orientated lipid bilayer phospholipid head groups was investigated using ^{31}P solid state NMR. Lipid stock solutions were prepared in chloroform as follows: POPC at 100 mg/mL, cholesterol at 20 mg/mL, POPE at 100 mg/mL and POPG at 50 mg/mL. Lipids where subsequently combined in specific ratios to a combined weight of 10 mg to imitate different types of cellular membranes as shown in Table 3.3.

Table 3.3. Lipid combinations used to form macroscopically orientated lipid bilayers for ^{31}P solid state NMR spectroscopy.

Model membrane mimic	Lipid combination	Ratio (mol/mol)
Mammalian	POPC:cholesterol	7:3
Bacterial (Gram-positive)	POPE:POPG	1:3
Bacterial (Gram-negative)	POPE:POPG	3:1

Peptides where dissolved in methanol to a final concentration of 1.00 mg/mL and then added to the lipid combinations resulting in a final ratio of 1:50 peptide to lipid (mol/mol). Negative

control lipid samples only received methanol. The resulting suspension was brought to 400 µL with chloroform to ensure all peptides and lipids were dissolved. Nitrogen gas was then utilised to decrease the volume after which each solution was placed and dried in approximate equal proportions on three separate 18 mm × 9 mm glass slides. The slides where then placed in a lyophiliser overnight to ensure that all solvent was removed. Subsequently the slides where transferred into a humidity chamber kept at 90% humidity by a saturated solution of KCl. The following day, slides with corresponding lipid/peptide combinations where stacked and again left in the humidity chamber overnight. Subsequently, the stacked slides where wrapped in Teflon tape before being sealed in plastic.

^{31}P solid-state NMR measurements were performed at 270 K, 290 K and 310 K on a 300 MHz Advance spectrometer (Bruker, Rheinstetten, Germany) using a static triple resonance probe. To record phosphorous-31 spectra, a Hahn echo pulse sequence was applied using a ^{31}P B1 field of 50 kHz and an echo time of 10 µs with continuous wave proton decoupling.

3.3.12 Electrophysiological analysis of lipid bilayer pore formation

Electrophysiological analysis was used to study the pore forming capabilities of the purified tryptocidine C and commercial bacitracin A in planar lipid bilayer membranes. These experiments were done in most part by our collaborators Jan C. Behrends and research team at the University of Freiburg, Germany. An Orbit 16 chip-based electrophysiology setup from Nanion Technologies (Munich, Germany) was used to automatically form planar lipid bilayer membranes (total lipid concentration of 10 mg/ml) on MECA chips after the measurement chamber was filled with 150 µL of measurement buffer (150 mM $CaCl_2$, 10 mM Tris, 1 mM EDTA, pH 7.4). POPC:cholesterol and POPE:POPG lipid combinations were prepared in octane in similar mol/mol ratios as depicted in Table 3.3. The stability and efficiency of the bilayers were examined before experiments by at least 3 cycles of electroporation and reformation. Subsequently the membranes stability at 100 mV was also

tested for 3 minutes. After lipid stabilisation confirmation, single channel recordings were done at a membrane potential of 100 mV while the current signals were filtered at 500 Hz for Axopatch amplifier (Molecular devices, California, USA) recordings. Peptide stock solutions (100 µg/mL in 50% ethanol) were pre-diluted with measurement buffer before being added at the desired concentration in the measurement chamber. Single channel events were used to calculate mean pore size and construct current jump histograms.

3.4 Results and discussion

3.4.1 Isolation and purification of tryptocidine C

Br. parabrevis produces an antimicrobial peptide complex termed tyrothricin, which comprises of various analogues of the antimicrobial peptides tyrocidine and gramicidin. The assortment of tyrocidine analogues produced by this bacterium, most of which differ by one amino acid, makes the isolation and purification of single analogues a daunting task [18]. However, a study conducted by Vosloo *et al*. [26] presented a method whereby culture media could be supplemented with specific amino acids to instigate a shift in the analogue profile, so as to produce greater amounts of specific analogues. Therefore, to aid in the isolation of tryptocidine C, an adaptation of the media supplementation method described by Vosloo *et al*. [26] was used. *Br. parabrevis* was cultured in media supplemented with 10 mM tryptophan to allow for production of predominantly tryptocidine C. ESMS spectra of the tyrothricin crude extract, depicted in Figure 3.1 A and B, indicated that it contained predominantly tryptocidine B (Tpc B), tryptocidine C (Tpc C) and tryptocidine C_1 (Tpc C_1) (Figure 3.2). The extract also contained gramicidins (Figure 3.1 A). Furthermore, it can be seen from the ESMS signal intensities that tryptocidine C was the major analogue produced and extracted (Figure 3.1 B). Taken together, this indicates the success of the media supplementation culturing method, which in turn allows for less cumbersome purification methodology.

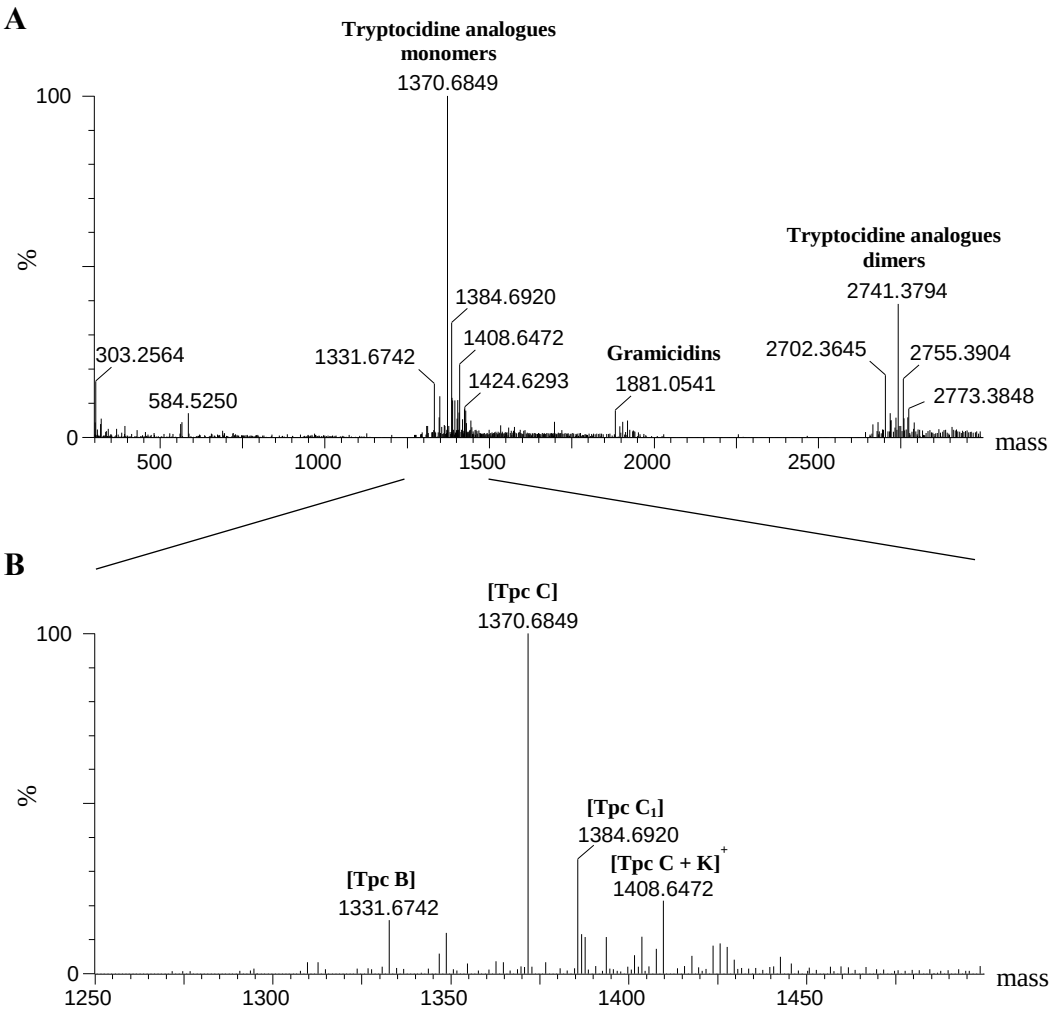


Figure 3.1. ESMS spectra following MaxEnt analysis of the crude tryptocidine C rich extract from a *Br. parabrevis* culture. (A) The ESMS spectrum shows the monomeric and dimeric species of tryptocidine analogues, as well as gramicidins. (B) The enlarged spectra displaing the M_r of different monomeric peptides in the extract. Tryptocidine is abbreviated as Tpc.

Various contaminants, including gramicidins, were partially removed from the crude extract by salting out followed by charcoal treatment and subsequent diethyl ether:acetone (1:1 *v/v*) wash steps. To remove all contaminants and purify tryptocidine C, the extract was subjected to semi-preparative RP-HPLC. As can be seen from Figure 3.2, fractions were only collected between 9 and 14 minutes (10 seconds per fraction) resulting in a total of 30 fractions. The range was selected as it flanked the peak at 254 nm which represents tryptocidine C, as determined by ESMS analysis (data not shown). The identity and signal intensity contribution of the peptides in each fraction was subsequently determined by ESMS, so as to identify

which fractions should be pooled to provide the highest purity as well as enough dry weight to perform further analysis. The mass spectrum was analysed by the MaxEnt algorithm in the MassLynx V4.1 software to obtain total ESMS signal intensities of each compound present in the fractions.

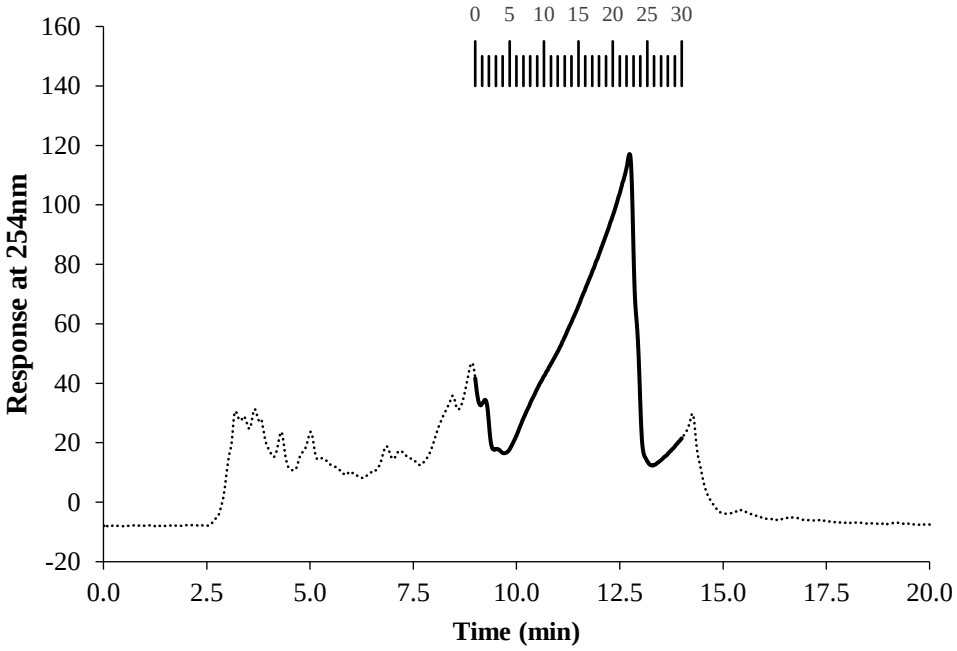


Figure 3.2. RP-HPLC chromatograph, visualized at 254 nm, of tryptocidine C purification. A total of 30 fractions were collected each with a fractionation time of 10 seconds. Fractions are indicated above the chromatogram and corresponds with the bold solid line of the chromatogram.

From Figure 3.3 A and B it is evident that the contribution of tryptocidine C to the total signal intensity was the highest in fractions 20, 21 and 22. These fractions were therefore pooled to obtain the highest purity, as well as a sufficient amount of tryptocidine C to perform further analysis. Thereafter, the analytical weight of the pooled sample was determined to be 3.453 mg which was sufficient for the subsequent investigations performed in this study. Interestingly, a small amount of tryptocidine C_1 was detected (results not shown), while tryptocidine B was also purified during RP-HPLC purification as indicated in Figure 3.3 A and C. This was the first time in our laboratory that tryptocidine B could be purified. Further analysis on tryptocidine B is, however, outside the scope of this study and

will only be investigated in future studies. The primary structures of these closely related tryptocidines are given in Figure 3.4.

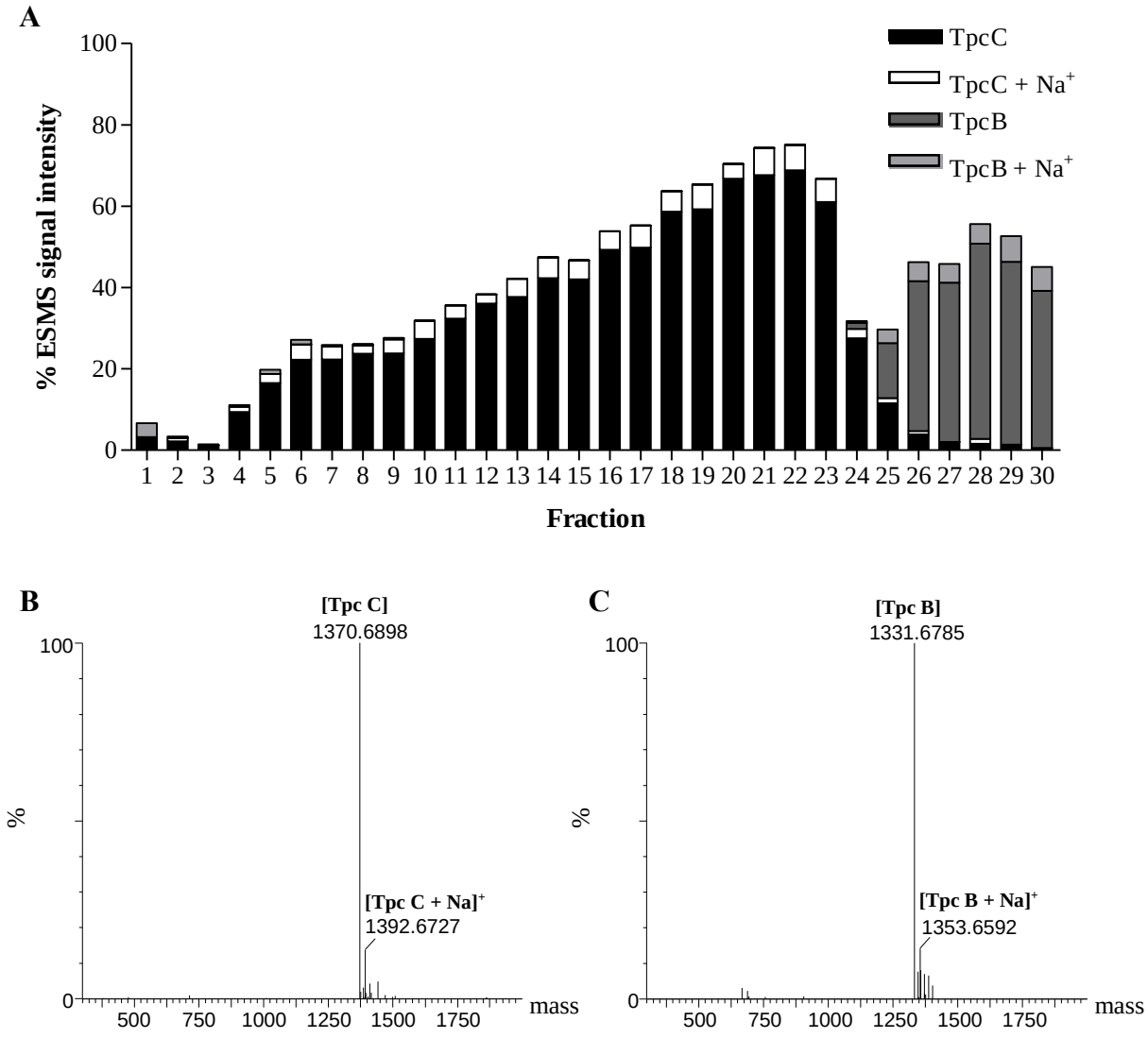


Figure 3.3. Percentage ESMS signal contribution of tryptocidine C, tryptocidine B and their sodium adducts towards the total ESMS signal intensity in each RP-HPLC fraction (A). Fraction 20, 21 and 22 displayed a high percentage tryptocidine C contributions whereas fraction 28 and 29 displays a high percentage tryptocidine B contribution. ESMS analysis followed by MaxEnt analysis of the fractions with the highest percentage tryptocidine C (B) and tryptocidine B (C) contribution displays the high purity obtained via RP-HPLC purification. Tryptocidine is abbreviated as Tpc.

UPLC-ESMS analysis indicated the final purity of the analytically weighed purified tryptocidine C to be > 95% (Figure 3.5). The small peak observed at the retention time of 11.15 was also observed in all blank samples (3% ACN) and was attributed to plasticisers

that leached from plasticware used in preparation of ESMS samples (Figure 3.5). Therefore, this peak was not included in purity calculations.

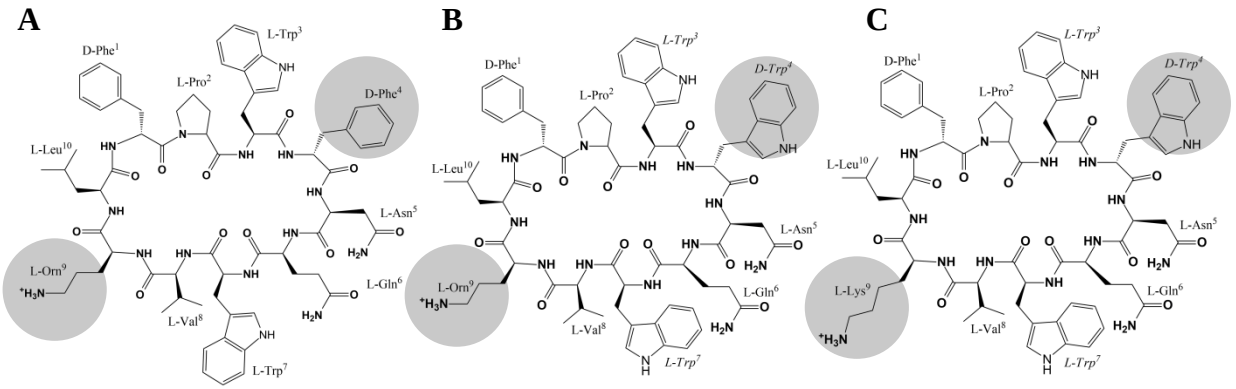


Figure 3.4. Primary structure of tryptocidine B (A), tryptocidine C (B) and tryptocidine C_1 (C). Standard three letter abbreviations are used to refer to residues with, Orn, representing ornithine.

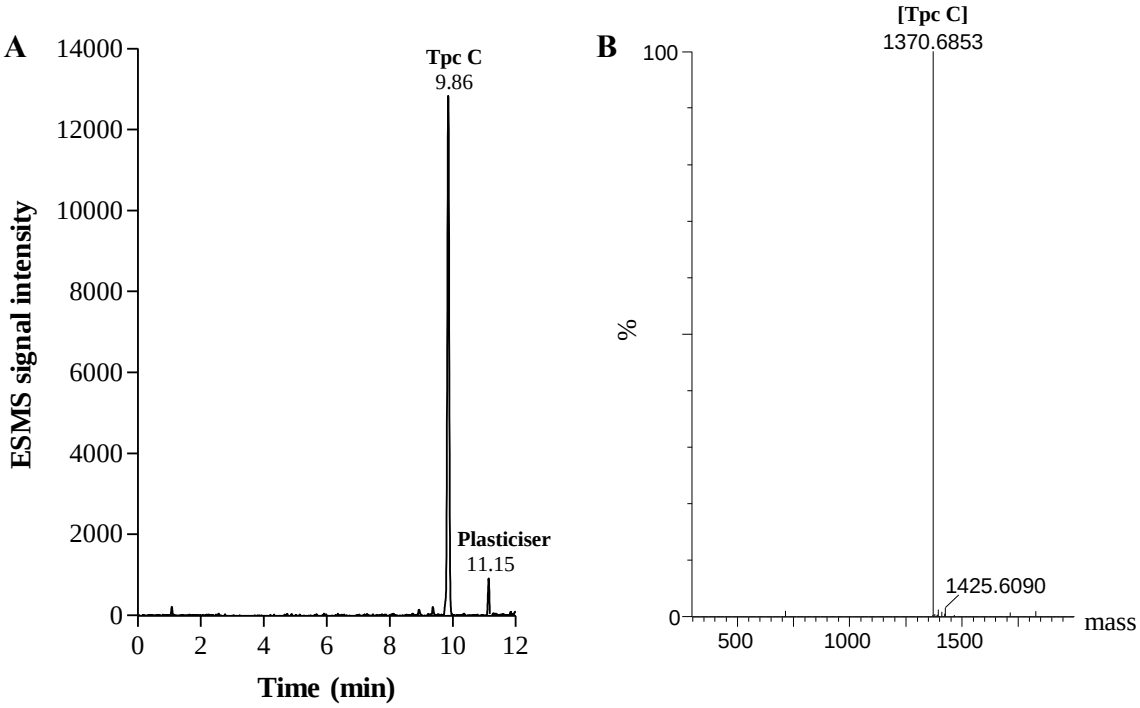


Figure 3.5. UPLC-ESMS analysis of purified tryptocidine C. (A) UPLC chromatogram of tryptocidine C (200 µg/mL) providing purity and retention time (in minutes). (B) ESMS spectrum showing M_r values from 300 to 2000 indicating the M_r and identity of purified tryptocidine C. Tryptocidine C is abbreviated as Tpc.

3.4.2 Isolation, identification and purification of the antimicrobially active compound(s) produced *B. licheniformis* LB.5

The isolate, *B. licheniformis* LB.5, was previously shown to possess antimicrobial activity towards the Gram-positive bacterium *M. luteus* (Chapter 2). Furthermore, from preliminary ESMS analyses it was inferred that the isolate produces the antimicrobial peptides, bacitracin A and bacitracin B (Chapter 2, Figure 3.12). Previous studies have established that bacitracins are secreted into the culture media of an *in vitro* culture [31]. Therefore, to isolate the active compound and confirm the hypothesis, an aerobically grown culture of *B. licheniformis* LB.5 was separated into cell pellet and supernatant fractions. Subsequently the supernatant was salted out with ammonium sulphate so as to precipitate the active peptide, which was collected by centrifugation. Following salting out, the antimicrobial activity of the crude extract (ACN washed precipitate) against *M. luteus* was investigated by spot-on-lawn analysis. A spot of 100 µg crude extract resulted in an inhibition zone of 305 ± 11 mm^2; a clear indication of the presence of an antimicrobial component (Table 3.4). Dissolving the extract in the working solvent (50% ACN) resulted in small amounts of fine insoluble debris which was removed by filtration with a 0.22 µm syringe filter. The antimicrobial activity towards *M. luteus* was again analysed to ensure that no or little antimicrobial activity was lost due to filtration. Following analysis inhibition zones of 290.9 ± 21 mm^2 were observed, thus indicating no substantial activity lost due to filtration (Table 3.4).

Table 3.4. *M. luteus* inhibition zone size produced by extracts of *B. licheniformis* LB.5.

Extract/control	**Area (mm^2) ± SD (*n*)**	**Diameter (mm) ± SD (*n*)**
crude extract	305 ± 16 (2)	19.3 ± 0.7 (2)
extract filtrate	291 ± 31 (2)	18.9 ± 0.5 (2)
gramicidin S (control)	104 ± 14 (2)	11.2 ± 0.6 (2)

Biological repeats are represented as *n*.

ESMS analysis of the filtrate again indicated the presence of compounds with M_r values of 1407.7390 and 1421.7493 (Figure 3.6), supporting the hypothesis that the antimicrobial compound produced by the isolate *B. licheniformis* LB.5 may be bacitracin A (M_r = 1421.7489) and bacitracin B (M_r = 1407.7333). However, ESMS analysis also displayed two other compounds (M_r values of 1519.7113 and 2487.0479) that possess significant signal intensities to be considered as antimicrobial candidates (Figure 3.6), necessitating further purification and identification.

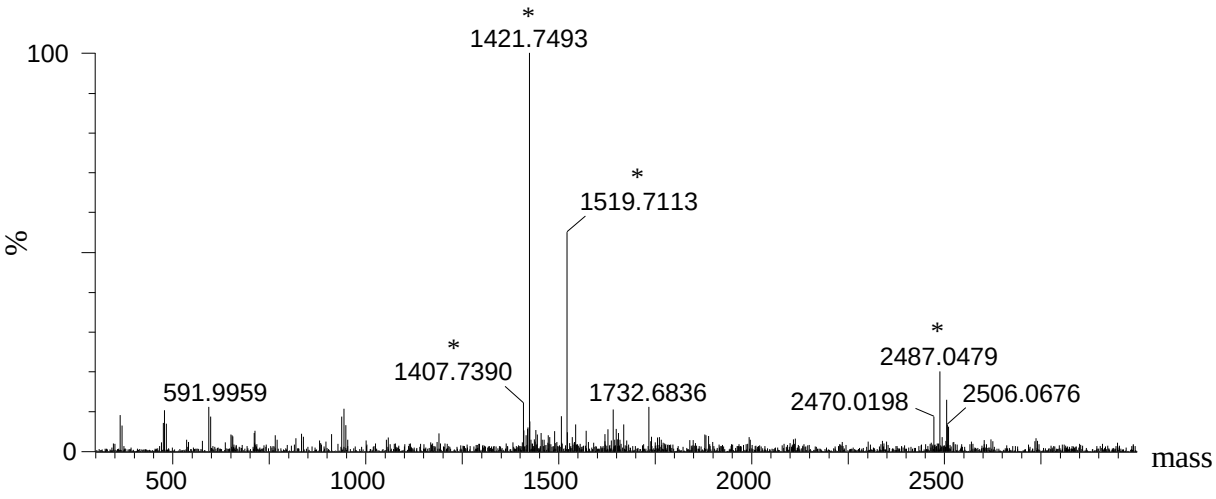


Figure 3.6. ESMS spectra analysed using the MaxEnt algorithm displaying the M_r values between 300 and 3000 of the *B. licheniformis* LB.5 crude extract. The * above peaks (1421.7493, 1519.7113 and 2487.0479) are indicative of a significant ESMS signal intensity so as to be suspected of providing the antimicrobial characteristic to the extract. Note that despite the low ESMS signal intensity of the M_r value 1407.7390, it was included as an antimicrobial suspect due to its similarity to the M_r of bacitracin B (1407.7333)

Definite identification of the antimicrobial compound(s) produced by *B. licheniformis* LB.5 was done by purifying the antimicrobial compound(s) from the filtrate using semi-preparative RP-HPLC. The eluent was separated into fractions between 2 and 16.5 minutes (10 seconds per fraction) resulting in a total of 87 fractions collected (Figure 3.7). To identify fractions that contained the active compound(s), the presence of antimicrobial activity towards *M. luteus* in each fraction was determined using spot-on-lawn analysis. The analysis indicated that only 4 fractions resulted in inhibition zones. These fractions were attributed to the RP-

HPLC peak at 254 nm with retention time of 11.18 minutes indicative of a more hydrophobic antimicrobial compound (Figure 3.7). This further strengthens the hypothesis as bacitracin is known to be amphipathic [31].

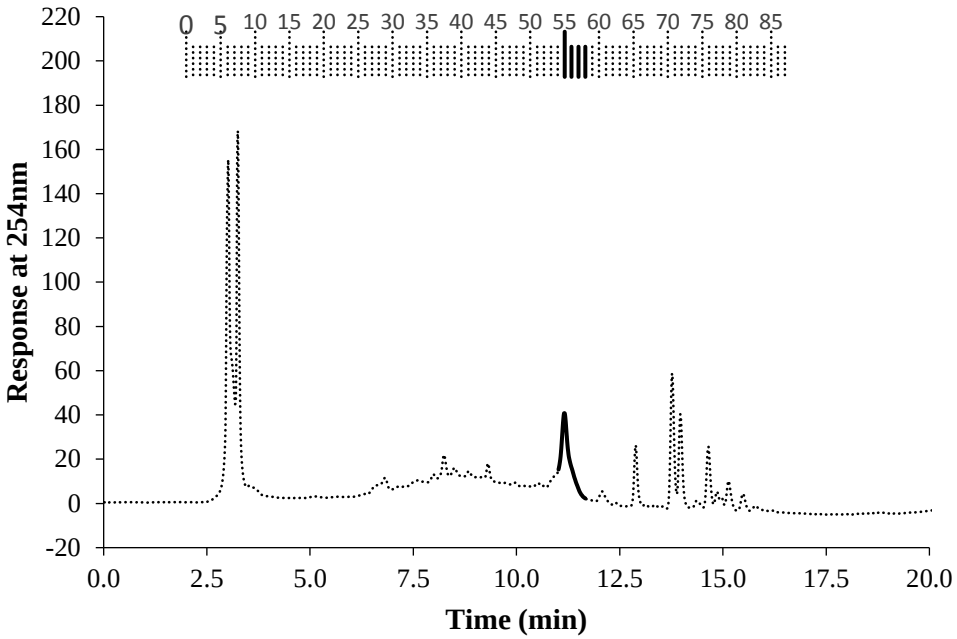


Figure 3.7. RP-HPLC chromatography of the *B. licheniformis* LB.5 extract filtrate visualised at 254 nm. Fractions were collected every 10 seconds from 2 to 16.5 minutes and are indicated by dotted lines above the chromatogram. Fractions that displayed antimicrobial activity towards *M. luteus* are indicated by the bold solid line/s on both the chromatogram and fraction lines.

To identify the active compound(s) in terms of M_r values, the isolated active fractions where subjected to ESMS analysis. This analysis clearly indicated that the M_r of the active compound was 1421.7478 (Figure 3.8 B). The hypothesis that *B. licheniformis* LB.5 produces bacitracin A and bacitracin B is therefore only partially supported due to the absence of the compound with a M_r value of 1407.7339, similar to that of bacitracin B. Additionally the analysis revealed which fractions contained the highest amount of the active compound (Figure 3.8 A). This gave an indication as to which samples should be pooled to obtain the highest purity and dry weight. From Figure 3.8 A, it is clear that the active compound contributed more than 50% of the total ESMS only in fractions 56, 57 and 58. These fractions

were pooled and analytically weighed before being subjected to UPLC-ESMS and UPLC-ESMS/MS analysis.

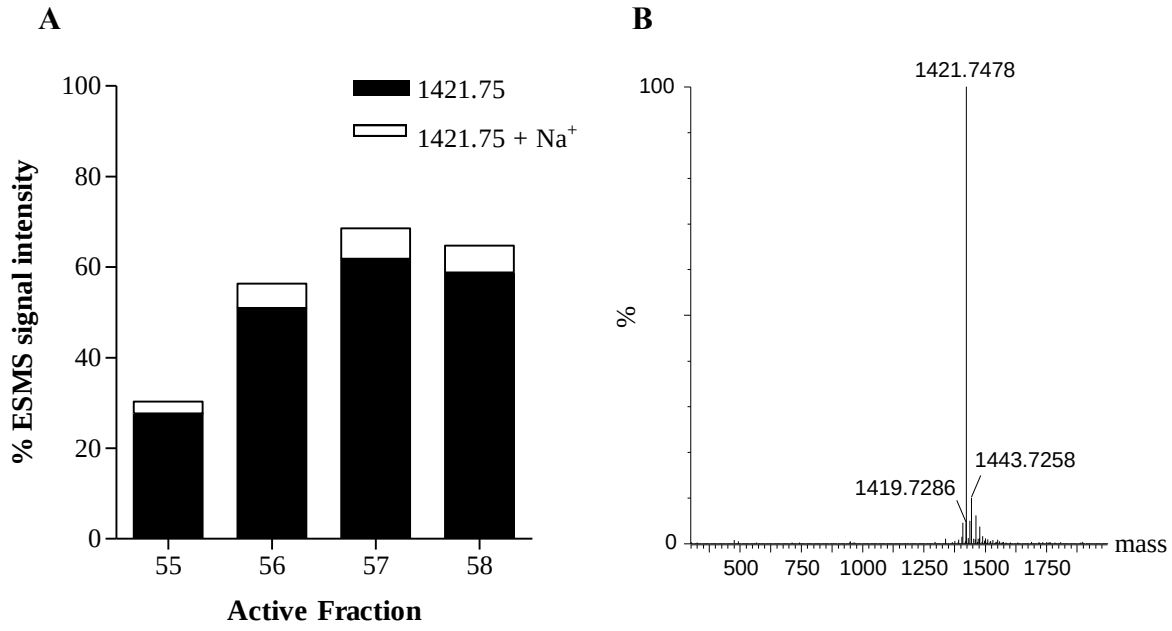


Figure 3.8. Analysis of the RP-HPLC fractions from the *B. licheniformis* LB.5 extract filtrate. A bar graph showing the percentage contribution of the active compound, identified to have a M_r of 1421.7478, and its sodium adduct in each of the RP-HPLC fractions that displayed antimicrobial activity (A). ESMS spectrum (analysed by the MaxEnt algorithm) of fraction 57 displaying the M_r of the active compound and the high purity obtained from semi-preparative RP-HPLC (B).

UPLC-ESMS analysis of the pooled fractions revealed that the compound with a M_r of 1421.7465 eluted from the UPLC column at 2.79 minutes (Figure 3.9). The purity of the sample was calculated to be > 95%. Commercial bacitracin A (71.1% HPLC purity) was also subjected to identical UPLC-ESMS analyses so as to compare it with the antimicrobial compound produced by the *B. licheniformis* LB.5 isolate, which is suspected to be bacitracin A. Figure 3.10 shows the commercial bacitracin A with a M_r of 1421.7448 eluting at 2.79 minutes. Thus, the commercial bacitracin A and the antimicrobial compound isolated from *B. licheniformis* LB.5 have identical M_r values (considering the ≤ 10 ppm mass error of the analytical method) as well as identical UPLC retention times (Figure 3.9 and Figure 3.10).

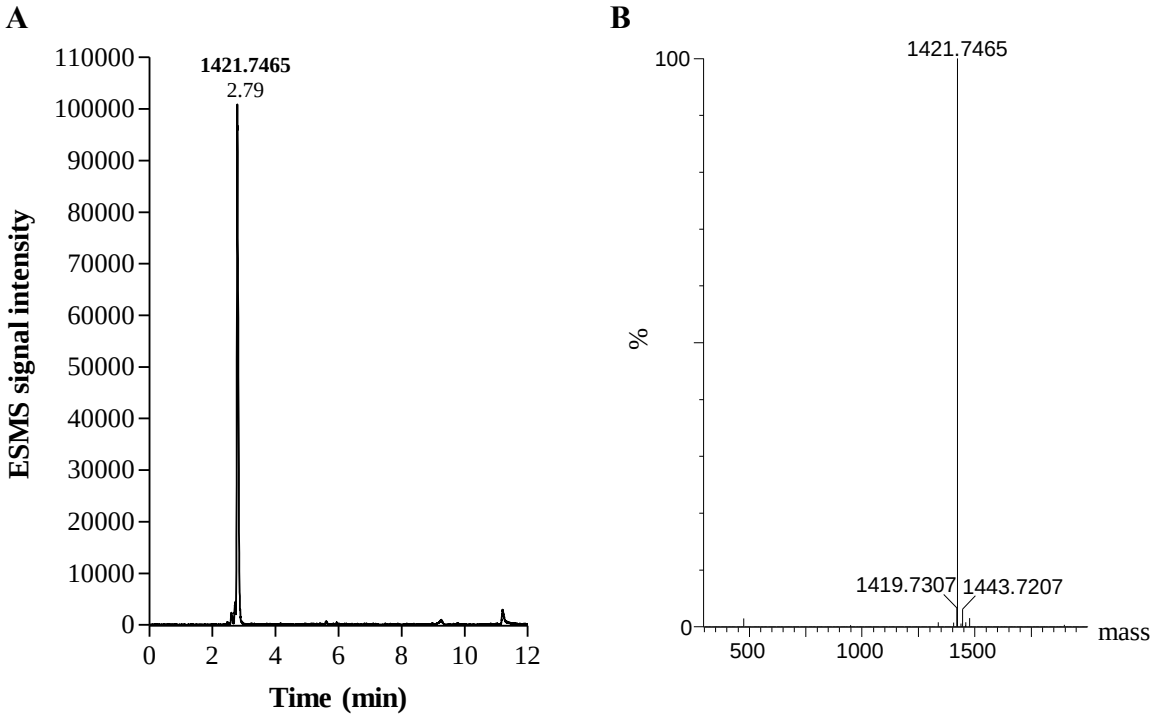


Figure 3.9. UPLC-ESMS analysis of the purified antimicrobial compound produced by *B. licheniformis* LB.5 (A). ESMS mass spectrum following MaxEnt analysis indicating the M_r of the purified antimicrobial compound produced by *B. licheniformis* LB.5 (B).

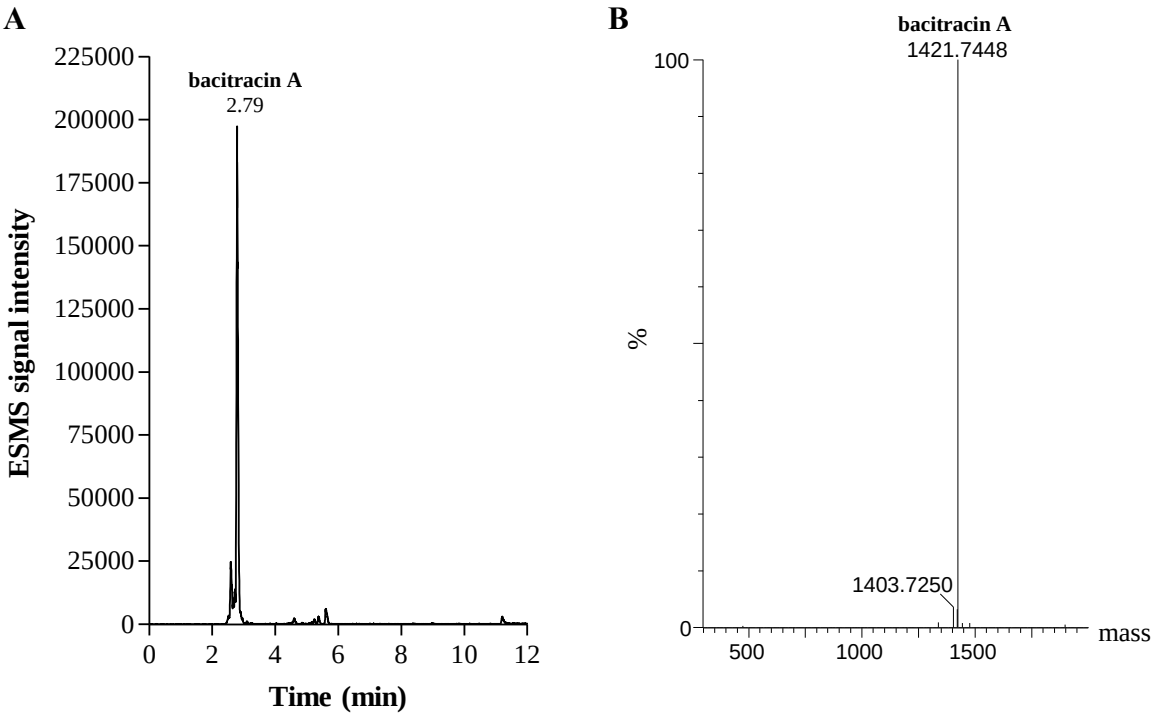


Figure 3.10. UPLC-ESMS analysis of commercial bacitracin A (A). ESMS mass spectrum following MaxEnt analysis indicating the M_r of commercial bacitracin A (B).

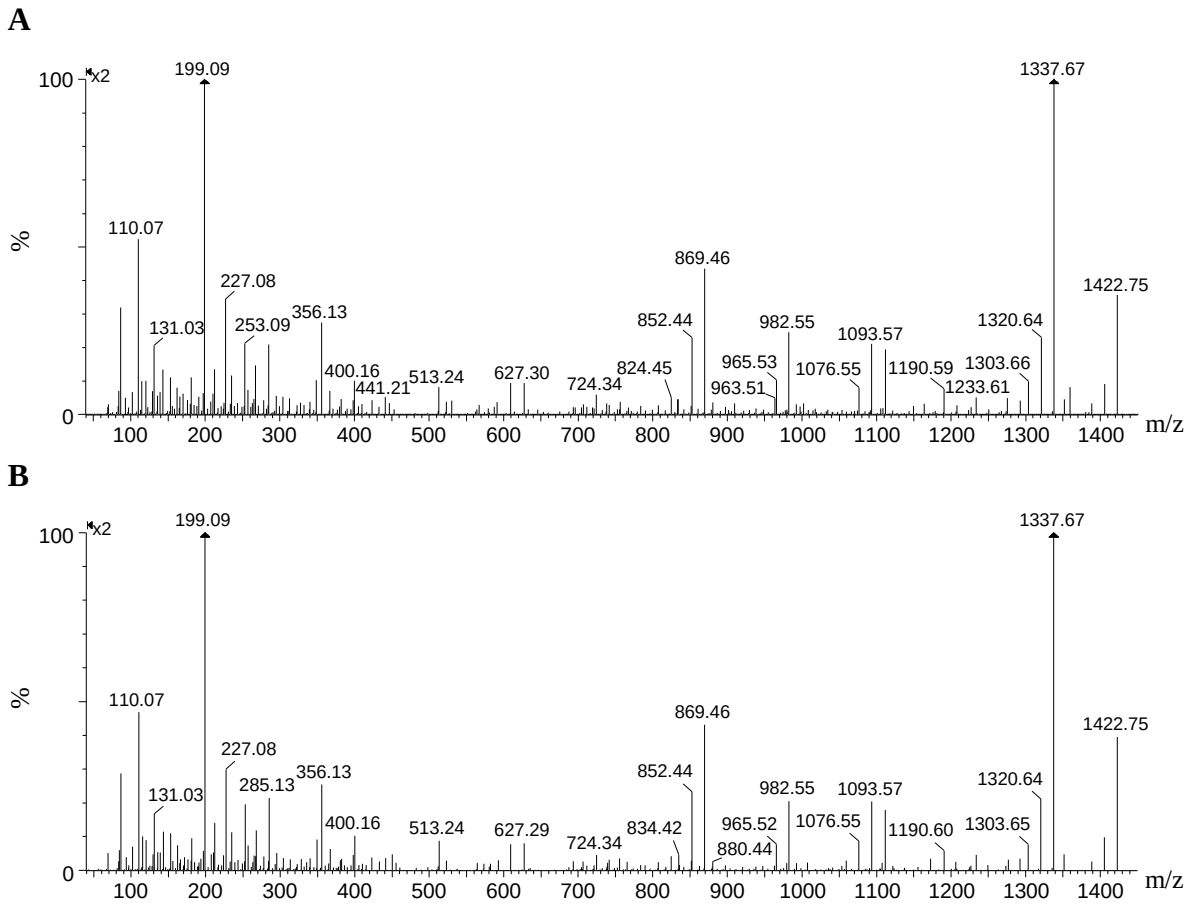


Figure 3.11. ESMS/MS spectra of the triply charged ($[M + 3H]^{3+}$) parent ion (*m/z* 474.90) of (A) the purified antimicrobial compound produced by *B. licheniformis* LB.5 and (B) commercial bacitracin A, displaying identical fragmentation profiles.

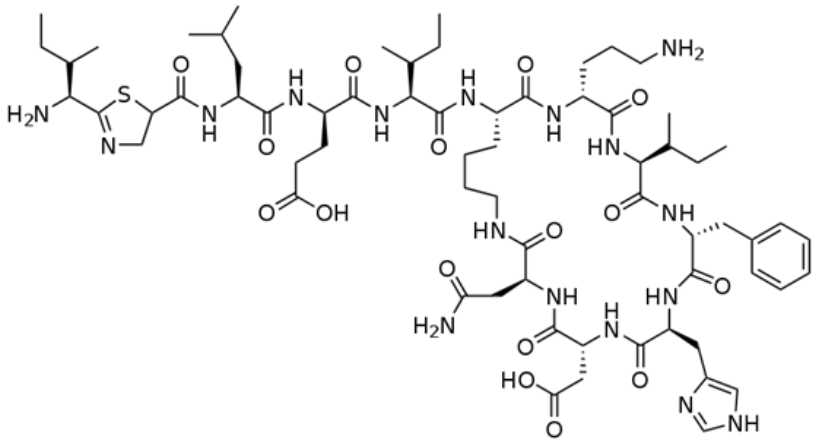


Figure 3.12. Primary structure of bacitracin A.

Furthermore, UPLC-ESMS/MS analysis of the isolated compound and commercial bacitracin A produced spectra with identical fragments (Figure 3.11). The primary structure of bacitracin A, as shown in Figure 3.12, has previously been determined. Therefore, no further analyses on the fragmentation profiles were done.

These results together with the strain identification of the LB.5 isolate as *B. licheniformis* (Chapter 2), a known producer of bacitracin, unequivocally confirmed that the antimicrobial compound produced by the *B. licheniformis* LB.5 isolate was indeed bacitracin A [31,32]. Further investigation on bacitracin A in this study included: bio-activity assays, NMR studies and electrophysiological studies.

The positive identification of bacitracin A production by *B. licheniformis* LB.5, as previously hypothesised (Chapter 2), demonstrated the practicality and efficacy of the ESMS peptide profile identification method developed and described in Chapter 2. It is therefore recommended that this methodology be used in future studies for a quick and efficient identification of antimicrobial peptides produced by soil isolates and other bacterial isolates. This will be especially beneficial for antimicrobial discovery, as it can be used to eliminate bacterial isolates that produce previously characterised antimicrobial peptides from the screening process. Furthermore, if a peptide is detected that has been previously characterised, established purification protocols can be implemented to decrease time spent on extraction and purification procedure optimization.

3.4.3 Isolation, identification and purification of the antimicrobial active compound(s) produced by *Br. laterosporus* LB.4

In Chapter 2 ESMS analysis indicated that *Br. laterosporus* LB.4 produced several compounds that are probable antimicrobial peptide candidates. Two of the compounds (M_r values of 1569.0597 and 1583.0856) were speculated to be the antimicrobial peptides BT1569 (M_r = 1569.0097) and BT1583 (M_r = 1583.1154) produced by *Brevibacillus*

texasporus, previously described by Wu *et al*. [16]. The M_r of the antimicrobial peptides produced by a strain of *Br. laterosporus*, bogorol A (M_r = 1583.0790) and bogorol B (M_r = 1569.0633), were also found to closely correlate with the compounds produced by *Br. laterosporus* LB.4 with M_r values of 1583.0856 and 1569.0597 respectively [17]. However, compounds with M_r values of 881.5872, 895.6042, 1272.6116 and 1222.6388 were also present in significant amounts as indicated by ESMS signal intensity (Chapter 2). The compounds with M_r values of 1272.6116 and 1222.6388 formed dimers during ESMS analysis, similar to antimicrobial peptides from the tyrocidine group, indicating that they had a tendency to aggregate or self-assemble. This may suggest that these two compounds may be self-assembling antimicrobial peptides. This prediction necessitated the isolation and purification of the antimicrobial compound(s) produced by *Br. laterosporus* LB.4 so as to identify the responsible compound(s).

To extract the antimicrobial compounds produced by *Br. laterosporus* LB.4, a combination of the methods used to extract tryptocidine C from *Br. parabrevis* (section 3.3.1) and bacitracin from *B. licheniformis* LB.5 (section 3.3.2) were used. Peptides from the cell pellet and peptides secreted in the supernatant were extracted in parallel in order to increase the likelihood of successfully and efficiently extracting antimicrobial compounds. An aerobically incubated culture of *Br. laterosporus* LB.4 was therefore separated into cell pellet and supernatant by centrifugation before extraction was conducted. The cell pellet was extracted with 50% ACN in water (*v/v*), whereas the supernatant was subjected to salting out by ammonium sulphate and subsequent precipitate collection by centrifugation. After lyophilisation, both extracts where dissolved in the 50% ACN working solvent. It was observed that the cell pellet extract still contained fine insoluble debris which was then removed by centrifugation. The supernatant extract, however, resulted in two liquid phases which was separated into the supernatant top phase and supernatant bottom phase. Again

centrifugation was used to remove fine insoluble debris in both the top and bottom phases of the supernatant extract. All three extracts where then tested for the presence of antimicrobial activity towards *M. luteus* using the spot-on-lawn method. From Table 3.5 it is evident that only the top phase of the supernatant extract and the cell pellet extract contained antimicrobial activity towards *M. luteus*. Furthermore, it was observed that the cell pellet extract resulted in a slightly larger inhibition zone of 131.3 ± 12.3 mm^2 as compared to the inhibition zone of the supernatant top phase extract of 91.9 ± 16.3 mm^2 (Table 3.5). This may be indicative of larger amounts of the antimicrobial compound(s) present in the cell pellet extract. Alternatively, the increased activity may be attributed to the presence of different antimicrobial compound(s) in the cell pellet extract which have higher activity towards *M. luteus*.

Table 3.5. *M. luteus* inhibition zone size produced by *Br. laterosporus* LB.4 extracts

Culture extract	**Area (mm^2) ± SEM (n)**	**Diameter (mm) ± SEM (n)**
supernatant top phase	91.9 ± 16.3 (6)	10.4 ± 0.9 (6)
supernatant bottom phase	No zone	No zone
cell pellet	131.3 ± 12.3 (9)	12.4 ± 0.6 (9)
gramicidin S	62.2 ± 15.9 (5)	8.2 ± 0.9 (5)

Biological repeats are represented by *n*.

ESMS analysis was done to establish whether the difference in inhibition zone size could be attributed to different molecular species and/or a concentration difference of the active compound(s) in the two extracts. The ESMS signal intensity contribution of compound(s) was used as a rough estimate of the concentration of each compound. It was observed that both the supernatant and pellet extracts contained approximately the same molecular species, including those suspected to be responsible for antimicrobial activity (Figure 3.13). However, from Figure 3.13 it can be seen that the compounds with M_r values of 1272.62 and 1222.64 displayed much greater contribution towards the total ESMS signal intensity in the cell pellet extract than in the supernatant top phase extract. Furthermore, percentage ESMS signal

intensity of the compounds with M_r values of 1569.06 and 1583.08, suspected to be BT1569 and BT1583 or bogorol B and bogorol A, were relatively small in both extracts (Figure 3.13). The compound with a M_r of 895.61 was also present in both extracts, however, it had a lower contribution towards the total ESMS signal intensity in the more active cell pellet extract (Figure 3.13 B). The supernatant top phase extract was not investigated further as it had lower antimicrobial activity towards *M. luteus* than the pellet extract. Furthermore, none of the antimicrobial compounds would be left out of this study by excluding this extract, due to the observation of similar M_r species in both the supernatant top phase and cell pellet extracts as determined by ESMS analysis (Figure 3.13).

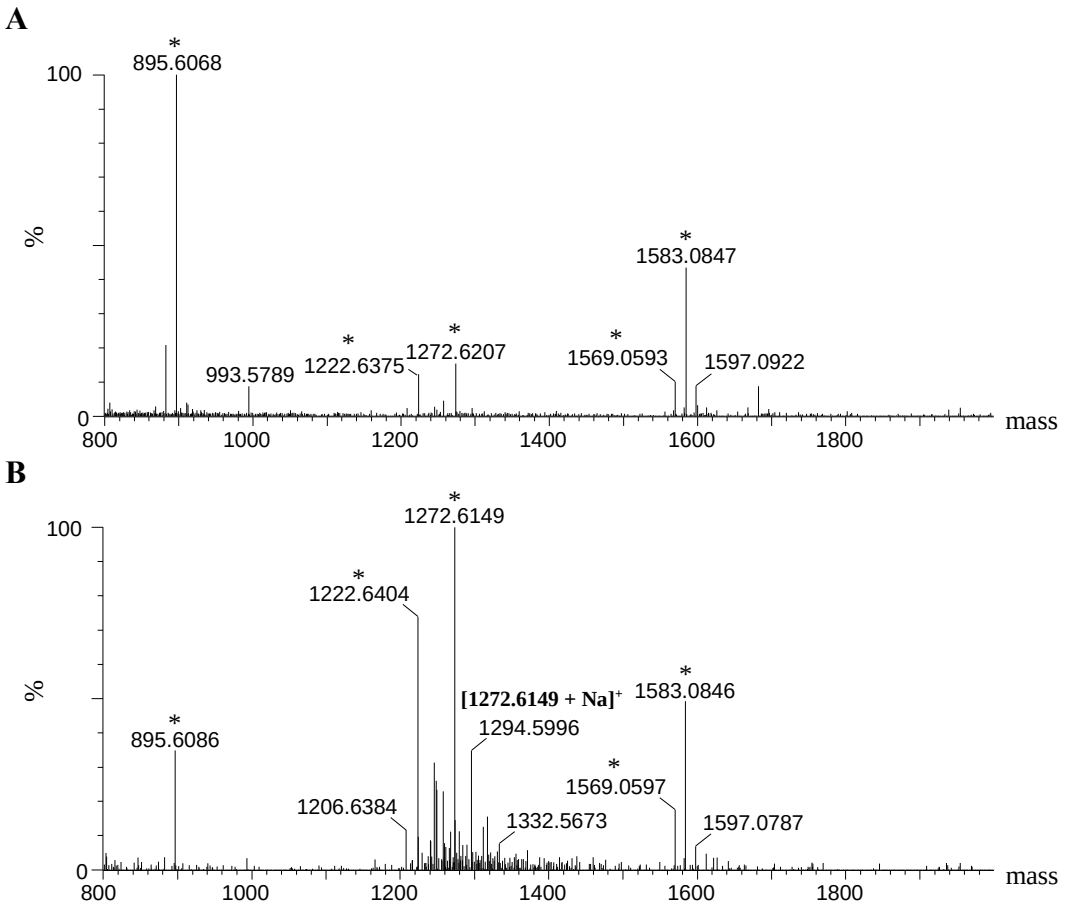


Figure 3.13. ESMS spectra from a M_r of 800 to 2000 following MaxEnt analysis of (A) the top phase of the supernatant and (B) cell pellet extracted from *Br. laterosporus* LB.4. The compounds hypothesized to be antimicrobial are indicated by * above their respective M_r values.

To identify the compounds with antimicrobial activity towards *M. luteus*, the cell pellet extract was first subjected to purification by RP-HPLC. Fractions were collected between 1.5 to 16 minutes (10 seconds per fraction) resulting in 87 fractions (Figure 3.14).

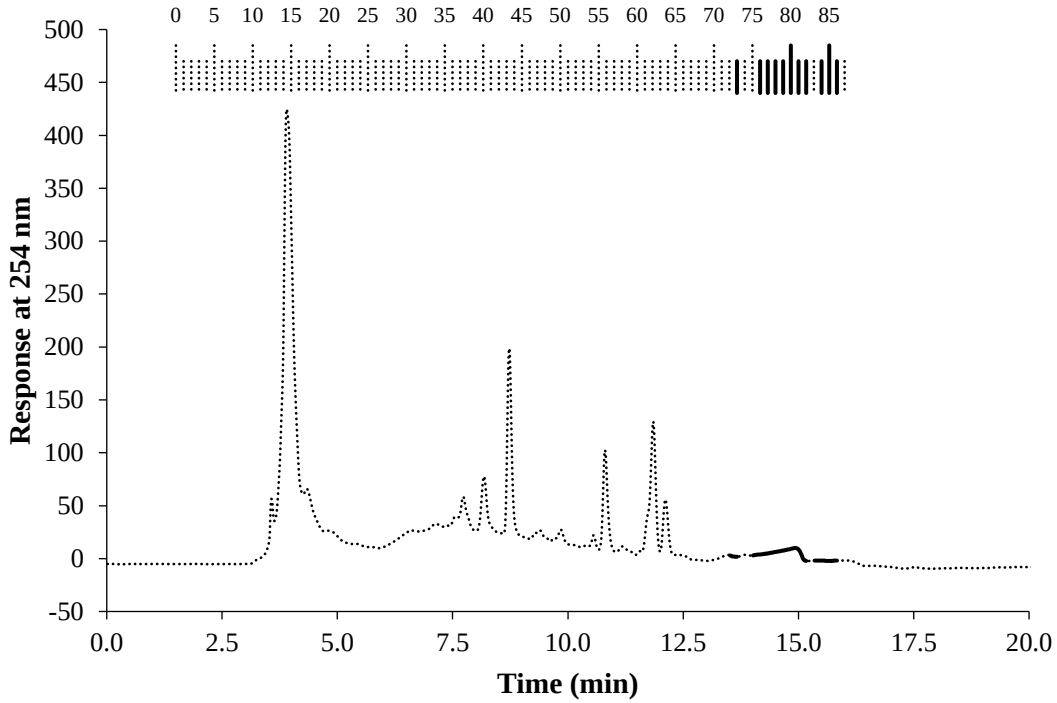


Figure 3.14. RP-HPLC chromatography of the *Br. laterosporus* LB.4 cell pellet extract visualised at 254 nm. Fractions collected from 1.5 to 16 minutes are indicated by the dotted lines above the chromatogram. Fractions that displayed antimicrobial activity towards *M. luteus* are indicated by the bold line/s on both the chromatogram and dotted fraction lines.

Following fractionation, each fraction was tested for its activity towards *M. luteus* using the spot-on-lawn method. This revealed that 11 fractions displayed antimicrobial activity towards *M. luteus* namely: 73, 76, 77, 78, 79, 80, 81, 82, 84, 85 and 86 (Figure 3.14). The late elution time ranging from 13.7 to 15.8 minutes is indicative of hydrophobic antimicrobial compound(s) (Figure 3.14). The poorly resolved peaks at 254 nm could be the result of either concentrations below the detectable limit, a lack of absorbance at the 254 nm or overt aggregation. Furthermore, the active fractions appear to be divided into three groups, separated by non-active fractions with group I containing fraction 73, group II fraction 76 to

82 and group III fractions 84 to 86 (Figure 3.14). This can be indicative of either low concentrations of the active compound or resolution between different antimicrobial compounds. However, prior to spot-on-lawn analysis the samples were dissolved in a set volume of 50% ACN rather than being made up to a pre-determined concentration. This was done due to the small amount of dry weight in each fraction and therefore the groupings could also have been a result of the overall sample concentration in the non-active fractions being less than the minimal concentration needed to produce inhibition zones.

ESMS analysis was subsequently conducted on all fractions ranging from fraction 73 to 87 to establish the identity of the antimicrobial compound(s) produced by *Br. laterosporus* LB.4 (Figure 3.15). Non-active fractions that separated active fractions, as described above, where also included in the analysis. The analysis revealed that the two different compounds with a M_r of 1222.6389 and 1272.6187 respectively, were responsible for antimicrobial activity towards *M. luteus* (Figure 3.15). Thus disproving the previous hypothesis that *Br. laterosporus* LB.4 produces either the antimicrobial peptides BT1569 and BT1583 or bogorol A and bogorol B. The active compounds with M_r of 1222.6389 and 1272.6187 were also present in fractions that did not display antimicrobial activity towards *M. luteus* (Figure 3.15 A). Therefore, indicating that the absence of antimicrobial activity might be due to a small amount of dry weight collected during fractionation. This would ultimately lead to a low amount of active compound being spotted on the *M. luteus* lawn and therefore resulting in no inhibition zone. The percentage signal intensity which each compound contributed towards the total signal intensity was calculated and used to give an indication of which samples to pool in order to obtain the highest purity as well as dry weight.

From Figure 3.15 it is evident that the compounds with a M_r value of 1222.6389 and 1272.6187 were purified during semi-preparative RP-HPLC. Only fractions 78, 79 and 80 where pooled due to their high percentage ESMS signal intensity of the compound with a M_r

of 1272.6187. Similarly, the pooled sample of the compound with a M_r of 1222.6389 consisted of fractions 84, 85 and 86. The two compounds with a M_r of 1272.62 and 1222.64 will be referred to as LB.4-1223 and LB.4-1273 in further discussions. Final purities of LB.4-1223 and LB.4-1273 were calculated from UPLC-ESMS analysis to be > 95% (Figure 3.16).

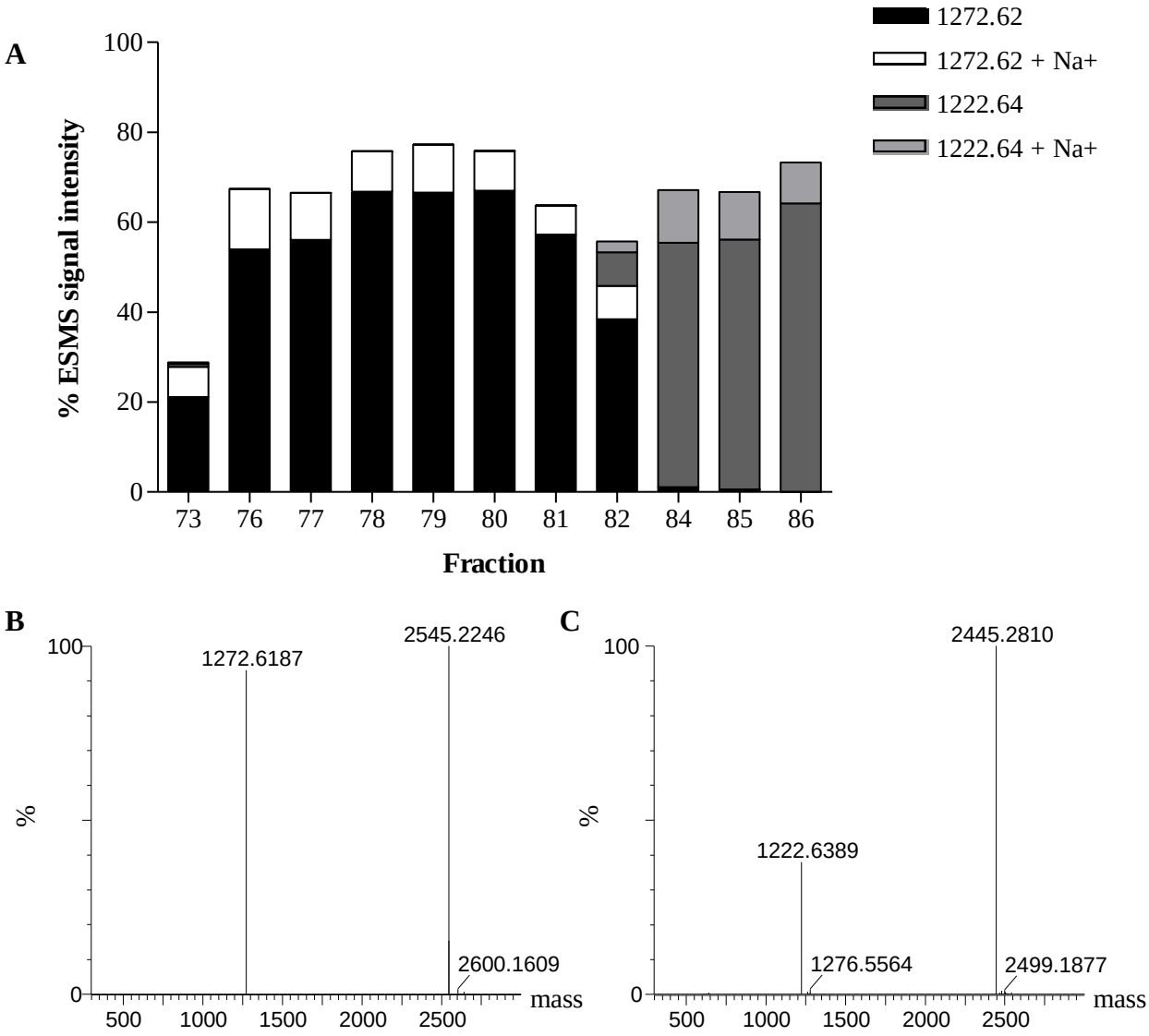


Figure 3.15. ESMS analysis of the antimicrobial active RP-HPLC fractions from the *Br. laterosporus* LB.4 cell pellet extract. Percentage contribution of the active compounds produced by *Br. laterosporus* LB.4 with M_r values of 1222.6389 and 1272.6187, and their sodium adducts in each fraction (A). ESMS mass spectra following MaxEnt analysis of the fraction with the highest percentage contribution of both the compounds with a M_r of (B) 1272.6187 (fraction 79) and (C) 1222.6389 (fraction 86). A high degree of dimerization was observed for both the compounds with a M_r of 1272.61 87(with dimers at 2545.2246) (B) and 1222.6389 (with dimers at 2445.2810) (C).

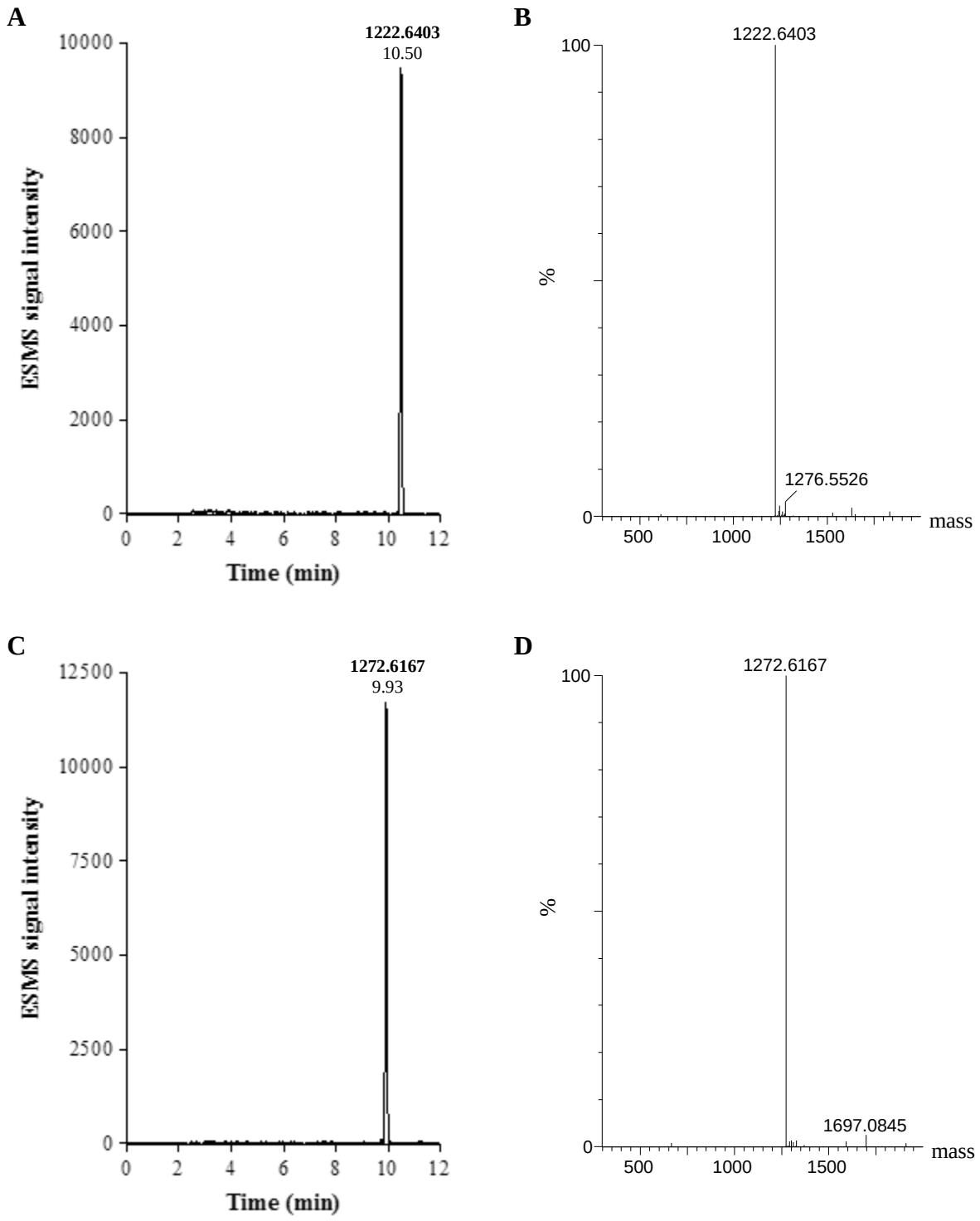


Figure 3.16. UPLC-ESMS analysis of purified (A) LB.4-1223 and (C) LB.4-1273. ESMS mass spectrum following MaxEnt analysis of (B) LB.4-1223 and (D) LB.4-1273.

This confirmed that these compounds were both responsible for the antimicrobial characteristics of the isolate *Br. laterosporus* LB.4. Furthermore, LB.4-1223 and LB.4-1273 were found to be highly hydrophobic with UPLC retention times of 10.50 and 9.93 minutes respectively (Figure 3.16 A and C). Interestingly the latter of the compounds had a retention time close to that of the highly hydrophobic antimicrobial peptide tryptocidine C (9.90 minutes) whereas LB.4-1273 was found to be even more hydrophobic (Figure 3.16 A and C, Figure 3.5). An extensive literature search did not reveal any antimicrobial agents with an identical or similar M_r values to either LB.4-1223 or LB.4-1273, thus, suggesting that these antimicrobial compounds may be novel.

Both compounds were also subjected to UPLC-ESMS/MS analysis in an attempt to elucidate their peptide sequence or aspects of their structure. The fragmentation spectra of the two compounds correlated closely with one another (Figure 3.17). A preliminary attempt to sequence LB.4-1273 revealed that the compound was indeed a peptide, which comprises of the amino acids; Pro, Leu/Ile, Asp/Asn, Phe, Val, Met, Tyr and possibly Gln (Table 3.6).

From the amino acids identified we were able to predict an amino acid composition of LB4-1273 as 2×Tyr, 1×Phe, 1×Pro, 2×Leu/Ile, 1×Met, 1×Val and 2×Asn that results in a M_r of 1272.6213 that is 4.6 ppm more than the observed M_r of 1272.6167. For LB4-1223 the amino acid composition was predicted as 1×Tyr, 1×Phe, 1×Pro 3×Leu/Ile, 1×Met, 1×Val and 2×Asn equivalent to a M_r of 1222.6420 that is only 1.7 ppm more than the detected M_r of 1222.6403. These amino acid compositions also correlate with the observed hydrophobicity of the two peptides. From the ESMS/MS fragmentation pattern of LB4-1273 short amino acid sequences, such as YLM, LMV, MV and LM, corresponding to YLMV, and FP, NFP and FPL corresponding to NFPL were predicted (Table 3.6). However, no amino acid sequence could be confidently ascribed to the fragments as the fragmentation patterns did not correspond to expected C-terminal fragments (y fragments) of linear peptides. Therefore,

further analysis of the fragmentation profile as well as amino acid sequence is still required to ultimately establish whether these antimicrobial peptides are indeed novel. Although the amino acid sequence and structure of these peptides has not yet been determined we can hypothesise that these may be novel peptides or peptides that have not been well-studied. This again confirms the usefulness of the ESMS peptide profile analysis described in Chapter 2 as an early screening method, to identify novel antimicrobial candidates.

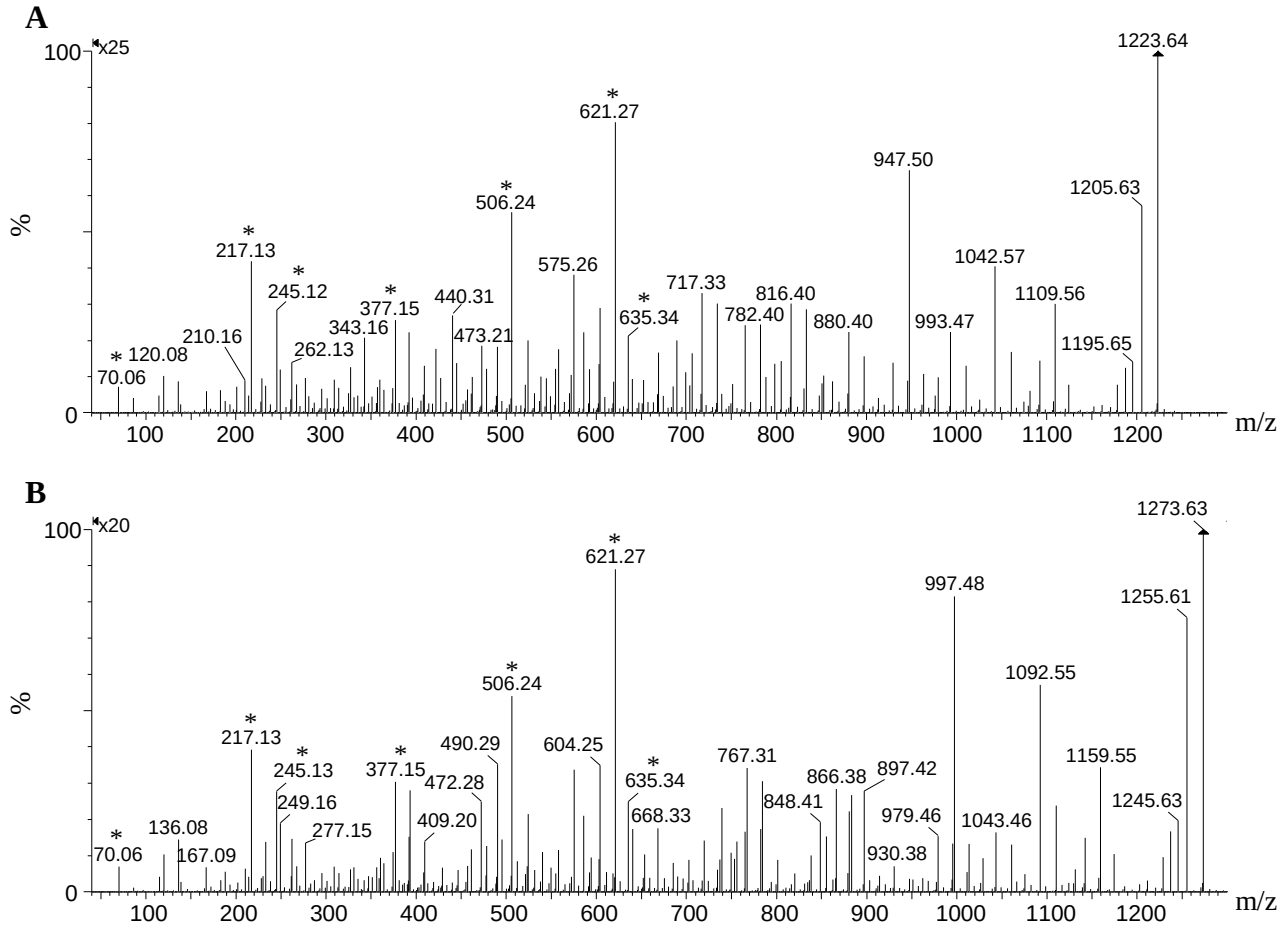


Figure 3.17. ESMS/MS fragment spectra of the singly charged ($[M + H]^+$) parent ions (A) *m/z* 1223.64 and (B) *m/z* 1273.62 of the purified antimicrobial compounds produced by *Br. laterosporus* LB.4. Similarities in the fragmentation profiles are indicated by *.

Table 3.6. Preliminary sequencing of LB.4-1273

M_r of major fragments		M_r difference [a]	Amino acid [b]
70.0646		70.0646	Immonium Pro
217.1327	70.0646	147.0681	Phe
	120.0803	97.0524	Pro
249.1591	136.0754	113.0837	Leu/Ile
377.1447	262.12	115.0247	Asp
392.1945	245.1273	147.0672	Phe
442.2302	343.1602	99.07	Val
462.2296	392.1945	70.0351	Immonium Pro
490.228	343.1602	147.0678	Phe
	377.1447	113.0833	Leu/Ile
506.2394	392.1945	114.0449	Asn
524.2124	377.1447	147.0677	Phe
576.2518	445.2065	131.0453	Met
604.2449	473.205	131.0399	Met
621.2657	490.228	131.0377	Met
	506.2394	115.0263	Asp
	524.2124	97.0533	Pro
622.2676	507.235	115.0326	Asp
689.3313	576.2518	113.0795	Leu/Ile
717.3276	604.2449	113.0827	Leu/Ile
718.3272	621.2657	97.0615	Pro
734.3506	621.2657	113.0849	Leu/Ile
735.3502	622.2676	113.0826	Leu/Ile
782.4063	635.3528	147.0535	Phe
805.4241	706.356	99.0681	Val
816.3926	717.3276	99.065	Val
817.3974	689.3313	128.0661	Gln
	718.3272	99.0702	Val
833.4197	734.3506	99.0691	Val
834.4179	706.356	128.0619	Gln
	735.3502	99.0677	Val
880.3925	717.3276	163.0649	Tyr
	765.3608	115.0317	Asp
897.4175	734.3506	163.0669	Tyr
947.4983	834.4179	113.0804	Leu/Ile
1060.5829	947.4983	113.0846	Leu/Ile
1273.629	1110.5732	163.0558	Tyr

[a] M_r difference was determined by the subtraction of the M_r values of various fragments observed by ESMS/MS analysis from one another.
[b] Amino acids were allocated based on their M_r as compared to the calculated M_r difference between fragments

3.4.4 Antimicrobial and haemolytic activity characterisation

Micro-broth dilution antimicrobial assays were done to assess the antimicrobial activity of all of the purified peptides towards the Gram-positive bacterium *M. luteus*. The well-known antimicrobial peptide, gramicidin S, was used as a positive control. Gramicidin S displayed similar activity towards *M. luteus* (IC_{50} of 5.6 µM) as previously reported [23,33] (Figure 3.18 A and Table 3.7). From Figure 3.18 A and Table 3.7 it can be seen that bacitracin A had a much greater activity towards *M. luteus* than all the other peptides, with an IC_{50} of 1.4 µM. Tryptocidine C and the isolated peptide LB.4-1273 had similar antimicrobial activity with IC_{50} values of 13 µM and 15 µM respectively (Table 3.7). Interestingly, the peptide LB.4-1223 had much lower activity towards *M. luteus* than its co-produced peptide LB.4-1273 with an IC_{50} of 28 µM (Table 3.7). The IC_{max} of these peptides (Table 3.7) also followed a similar trend as the IC_{50} (bacitracin A > gramicidin S > tryptocidine C ~ LB.4-1273 > LB.4-1223).

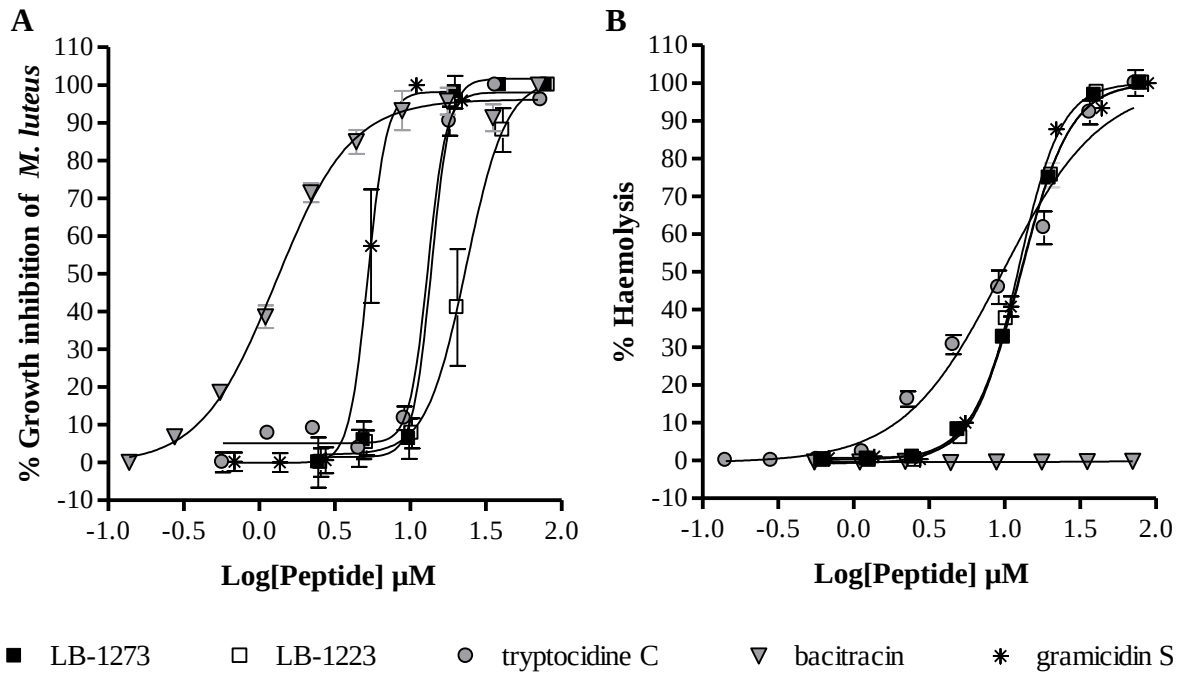


Figure 3.18. Dose response assays to elusidate the (A) antimicrobial activity towards *M. luteus* and (B) haemolytic activity of tryptocidin C, bacitracin A, LB.4-1223 and LB.4-1273. Each data point for the analysis of antimicrobial and haemolytic activity represents the mean ± SEM of 3 and 2 biological repeats respectively.

Table 3.7. Antimicrobial and haemolytic activities of the purified peptides and gramicidin S.

Peptides	Antimicrobial activity towards *M. luteus* (µM)		Haemolytic activity (µM)
	IC_{50} ± SEM (*n*)	IC_{max} ± SEM (*n*)	HC_{50} ± SD (*n*)
tryptocidine C	13.0 ± 0.4 (3)	17.7 ± 0.38 (3)	10.2 ± 4.00 (2)
bacitracin A	1.41 ± 0.06 (3)	4.02 ± 0.54 (3)	> 100 (2)
LB.4-1273	14.7 ± 0.60 (3)	19.7 ± 0.81 (3)	12.9 ± 0.59 (2)
LB.4-1223	27.6 ± 6.68 (3)	37.4 ± 9.11 (3)	12.8 ± 0.75 (2)
gramicidin S	5.64 ± 0.83 (6)	7.56 ± 1.12 (6)	12.06 ± 0.85 (2)

Biological repeats are represented by *n*.

As described earlier, both peptides isolated from *Br. laterosporus* LB.4 were highly hydrophobic in nature. Dissolving these peptides in 15% ethanol prior to adding the dilution series to the cell suspension resulted in an opaque emulsion that, if left undisturbed for an extended time period, would precipitate. This may have led to a lower concentration of the active peptide in the dilution series. Therefore, the IC_{50} values obtained could be an underestimation of the activity of these peptides towards *M. luteus*. Furthermore, the small difference between active and non-active peptide concentration seen for tryptocidine C, LB.4-1223 and LB.4-1273 (Figure 3.18 A) have previously been linked to the formation of inactive aggregates in solution, which could also result in a lower antimicrobial activity [30]. In future, more in depth activity studies will be focused at determining the optimal solvent for dissolving the peptides which also has minimal effect on the growth of *M. luteus* such as dimethyl formamide or dimethyl sulphoxide [34].

Bacitracin A has been used pharmaceutically for many years and is known not to be haemolytic [13,31]. On the other hand, haemolysis has been described previously to be a characteristic of all tyrocidine analogues which possess similar HC_{50} to that of gramicidin S [23,35]. Therefore, a haemolysis dose response assay was performed with all peptides so as to assess whether the newly isolated peptides (LB.4-1223 and LB.4-1273) possess haemolytic

activity and also to compare their activity to that of bacitracin A, tryptocidine C and gramicidin S in parallel. As expected, bacitracin A did not show any significant haemolytic activity (Figure 3.18 B and Table 3.7). From Table 3.7 it is evident that tryptocidine C had the highest haemolytic activity with an HC_{50} of 10.2 µM, correlating with results from a previous study and validating the assay [3]. Gramicidin S, LB.4-1223 and LB.4-1273 displayed similar haemolytic activity with HC_{50} of 12.4, 12.9 and 12.8 µM respectively (Table 3.7). Again the formation of an opaque suspension rather than a clear solution was obtained in the working solvent of 15% ethanol and therefore the HC_{50} values obtained for LB.4-1223 and LB.4-1273 could have led to an underestimation.

Comparing antimicrobial and haemolytic activity, it can be seen, as expected, that bacitracin A had specificity towards the Gram-positive bacterium *M. luteus*. This is supported by the non-lytic mode of action of bacitracin A, in which bacitracin A inhibits cell wall synthesis by interfering with the dephosphorylation of C_{55}-isoprenyl pyrophosphate [14]. However, tryptocidine C, LB.4-1223 and LB.4-1273 did not display an overt specificity towards either *M. luteus* or erythrocytes. Tyrocidine analogues have been previously suggested to act via a membranolytic mode of action [3,23,24]. The relatively non-specific targeting of the *Br. laterosporus* LB.4 peptides could, therefore, also be indicative of a membrane-mediated mode of action. Further studies, however, need to be done to investigate whether the two co-produced peptides isolated from *Br. laterosporus* LB.4 have synergistic or antagonistic activity when used in combination.

3.4.5 Mode of action and biophysical studies

Many antimicrobial peptides have been shown to exert their antimicrobial activity by targeting either intracellular targets and/or cellular membranes [36]. We have focused on elucidating the previously hypothesised membrane-disruptive mode of action of tryptocidine C in comparison with the non-lytic bacitracin A. The mode of antimicrobial action of

bacitracin has previously been extensively studied [14]. It has been ascertained that bacitracin acts by binding to C_{55}-isoprenyl pyrophosphate, a cell wall synthesis intermediate, and interfering with its dephosphorylation, in turn resulting in peptidoglycan synthesis inhibition [14]. Unfortunately no biophysical analysis on the mode of action of the two novel peptides, LB.4-1223 and LB.4-1273, could be done due to the small amounts of pure peptide that was available during a research visit to the University of Strasbourg and the University of Freiburg. These peptides will be subjected to in-depth biophysical analyses in future studies.

^{31}P solid state NMR has previously been used to study the binding of peptides to a lipid bilayer and the response of lipids to the binding in order to elucidate mode of action on membranes [37,38]. ^{31}P solid state NMR analysis provides an indication of the macroscopic orientation of phospholipid head groups in the presence of antimicrobial peptides. The lipid orientation in the presence of peptide can reveal whether the peptides act via the membrane, as well as its specific membrane interaction and disruption mechanism such as the effect of the peptides on membrane fluidity or pore formation [38].

The effects that the antimicrobial peptides, bacitracin A and tryptocidine C, has on three model lipid membranes, POPC:cholesterol (7:3), POPE:POPG (1:3) and POPE:POPG (3:1), were investigated in collaboration with the Bechinger group at the Department of Biophysics, University of Strasbourg, France. Three model lipid membranes were used so as to mimic the cellular membranes of eukaryotes, Gram-positive and Gram-negative bacteria respectively. Macroscopically orientated lipid bilayers in the presence and absence of the antimicrobial peptides (2% mol/mol) were prepared on thin glass slides. The ^{31}P solid state NMR spectra were then obtained at 270 K, 290 K and 300 K so as to study both lipid phase transition and orientation of the phospholipid head groups. The reference scale was based on 85% phosphoric acid set to 0 ppm.

Figure 3.19 indicates the effect bacitracin A and tryptocidine C has on the eukaryotic cellular membrane model, POPC:cholesterol (7:3), at all temperatures. In the absence of peptide a well-orientated POPC:cholesterol (7:3) lipid bilayer was obtained with a chemical shift at 26 ppm (Figure 3.19 A-I to A-III). Furthermore, at 270 K the lipid bilayer was observed to be in gel phase indicated by the broad line shape (Figure 3.19 A-I). Phase transition was observed between 270 K and 290 K resulting in a sharp peak at 290 K and 310 K (Figure 3.19 A-I to A-III).

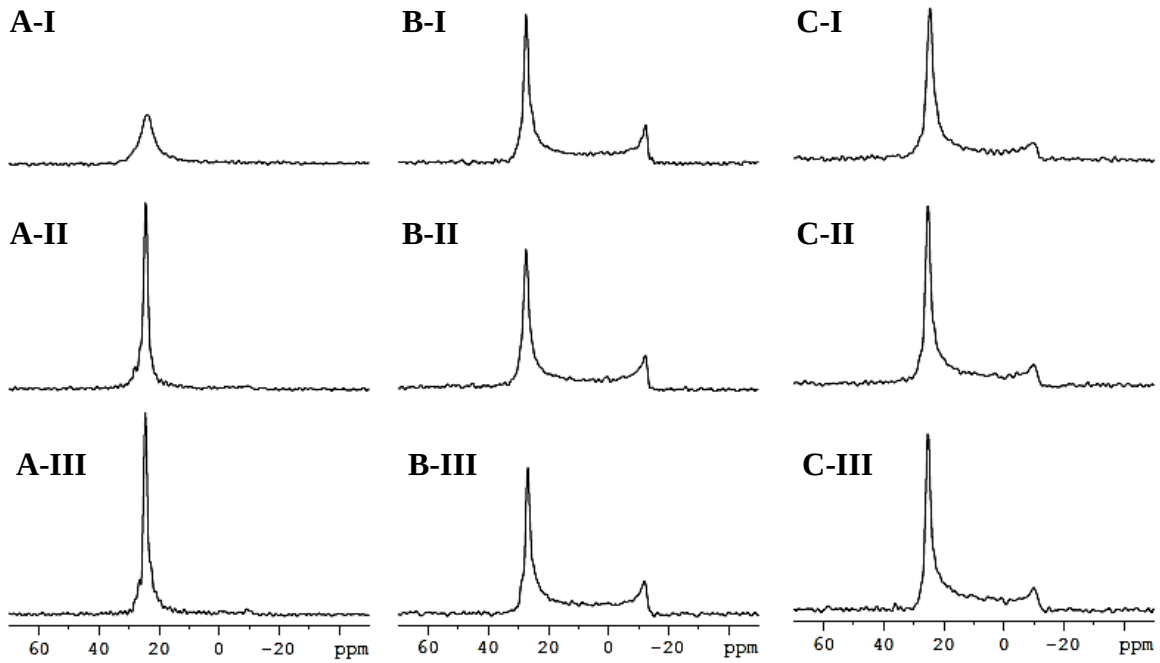


Figure 3.19. ^{31}P solid state NMR spectra of macroscopically orientated POPC:cholesterol (7:3) bilayers (eukaryotic cell membrane model) with (A) methanol, (B) bacitracin A and (C) tryptocidine C at (I) 270 K, (II) 290 K and (III) 310 K.

The addition of bacitracin A (2% mol/mol), unexpectedly, led to disorientation of phospholipid head groups observed at a chemical shift of -12 ppm in all temperatures (Figure 3.19 B-I to B-III). Furthermore, the line shape observed at 270 K, when bacitracin A was present, is indicative of the bilayer being in a liquid state (Figure 3.19 B-I). Thus, the phase transition of the POPC:cholesterol (7:3) bilayer was greatly affected by bacitracin A at 2% mol/mol, indicating membrane interaction. Similarly, tryptocidine C (2% mol/mol) also

resulted in a chemical shift of -12 ppm at all temperatures measured, indicative of disorientated phospholipid head groups (Figure 3.19 C-I to C-III). Similar to bacitracin A, tryptocidine C was also found to affect the phase transition of the (2% mol/mol) POPC:cholesterol (7:3) bilayer (Figure 3.19 C-I to C-III), indicating membrane interaction as expected.

^{31}P solid state NMR analysis of a macroscopically orientated POPE:POPG (1:3) lipid bilayer (Gram-positive bacteria cellular membrane model) in the absence of peptide was indicative of a well-orientated lipid bilayer (Figure 3.20 AI-AIII). At 290 K and 310 K the orientated bilayer was observed to be in liquid phase with a chemical shift of 29 ppm (Figure 3.20 A-II and A-III). ^{31}P solid state NMR spectra obtained at 270 K, however, was indicative of the bilayer transitioning between gel phase (chemical shift of 40 ppm) and liquid phase (chemical shift of 29 ppm) (Figure 3.20 A-I).

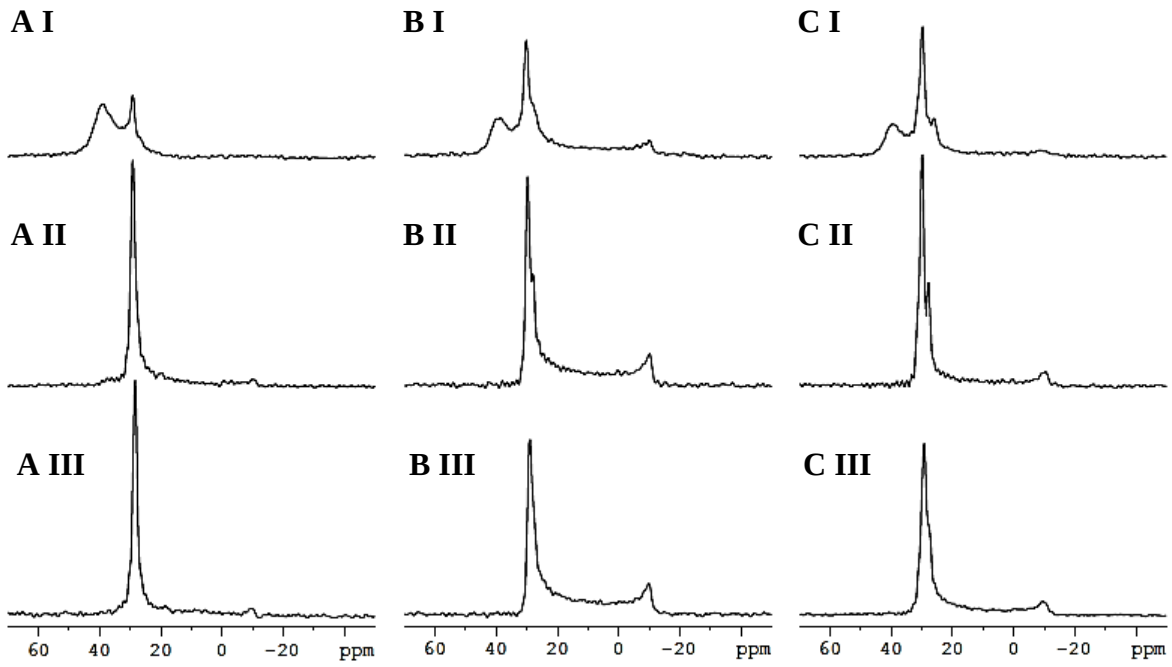


Figure 3.20. ^{31}P solid state NMR spectra of macroscopically orientated POPE:POPG (1:3) bilayers (Gram-positive bacteria cell membrane mimic) with (A) methanol, (B) bacitracin A and (C) tryptocidine C at (I) 270 K, (II) 290 K and (III) 310 K.

From Figure 3.20 B-I to B-III it is evident that the addition of bacitracin A (2% mol/mol) results in the disorientation of the phospholipid head groups at a chemical shift of -10 ppm in all temperatures measured. Tryptocidine C caused disorientation to a lesser extent and only at 290 K and 310 K (Figure 3.20 C-II and C-III). However, similar to results obtained for the lipid bilayer, POPC:cholesterol (7:3), both the peptides resulted in the decreased temperature needed for phase transition, as indicated by the larger peak at 270 K with a chemical shift of 29 ppm in the presence of peptide as compared to when the peptides were absent (Figure 3.20 B-I and C-I).

Orientated phospholipid head groups of POPE:POPG (3:1) lipid bilayers (Gram-negative bacteria cellular membrane model) were observed to be in gel phase at 270 K at a chemical shift of 35 ppm (Figure 3.21 A-I). In contrast with the previous lipid bilayers, transition from gel phase to liquid phase (chemical shift of 30 ppm) only commenced at 290 K with the POPE:POPG (3:1) bilayer, however, still being predominantly in the gel phase (Figure 3.21 A-II). The full liquid phase of the lipid bilayer was only observed at 310 K at a chemical shift of 30 ppm (Figure 3.21 A-III).

The addition of both bacitracin A and tryptocidine C resulted in almost undetectable disorientation of the lipid bilayer only at 310 K with a chemical shift of -12 ppm (Figure 3.21 B-III and C-III). From Figure 3.21 B-II and C-II it is evident, however, that as previously observed in different lipid combinations both peptides resulted in a decreased temperature needed for phase transition as indicated by the larger amounts of lipid being in liquid phase (chemical shift of 30 ppm). However, both the peptides effected the phase transition of POPE:POPG (3:1) bilayers to a lesser extent as compared to the other two model membranes.

Tryptocidine C has previously been hypothesised to have a membrane-mediated mode of action [3,23,24]. Results from ^{31}P solid state NMR experiments clearly supports this hypothesis due to the observation of both disorientated phospholipid head groups and phase

transition shifts in macroscopically orientated POPC:cholesterol (7:3), POPE:POPG (1:3) and POPE:POPG (3:1), which mimic the cellular membranes of eukaryotes, Gram-positive and Gram-negative bacteria respectively. The disorientation of the phospholipid head groups, however, was only observed to a small degree. This may be due to the peptide aligning in between the two lipid layers, resulting in only slight phospholipid head group disturbance and an increase in membrane fluidity. Further studies will, therefore, be focused on advanced solid state NMR experiments to help elucidate the membrane disturbance of the hydrophobic tail groups of the phospholipids [37,38].

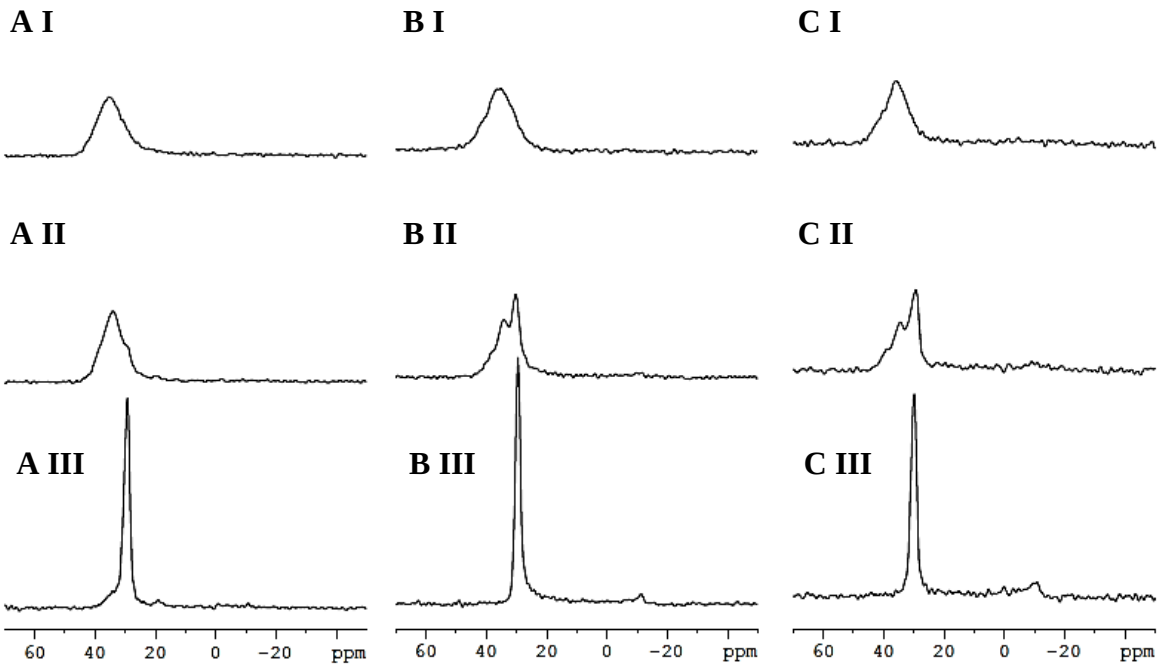


Figure 3.21. ^{31}P solid state NMR spectra of macroscopically orientated POPE:POPG (3:1) bilayers (Gram-negative bacteria cell membrane mimic) with (A) methanol, (B) bacitracin A and (C) tryptocidine C at (I) 270 K, (II) 290 K and (III) 310 K.

In contrast to tryptocidine C, the mode of bacitracin A's antimicrobial action has previously been extensively studied and determined to act predominantly by inhibiting peptidoglycan synthesis [14]. However, we have shown that bacitracin A does disturb the orientation of phospholipid head groups and influence the phase transition of macroscopically orientated POPC:cholesterol, POPE:POPG (1:3) and POPE:POPG (3:1). Furthermore, the disorientation

of the phospholipid head groups caused by bacitracin A was comparable to that of tryptocidine C, which have been previously shown to possess a membranolytic mode of action [3,23,24]. A study by Storm and Strominger [15] have shown the ability of bacitracin to disrupt the protoplasts of *B. licheniforms* and *M. luteus* (previously named *Micrococcus lysodeikticus*), however, no studies have reported on the mechanistic characteristics of the cellular membrane interaction of bacitracin A. This study, therefore, provides the first insights into the mechanistic characteristics of the interaction between bacitracin A and both eukaryotic and prokaryotic cellular membranes. However, whether the effects observed in phospholipid head group orientation and phase transition directly effects the antimicrobial activity of bacitracin A or if it only assists in transportation to its subcellular target still needs to be determined.

Ion channel electrophysiology experiments, done in collaboration with the Berhends group at the Institute of Physiology, University of Freiburg, Germany, was used to further investigate the membrane interactions of bacitracin A and tryptocidine C. This technique is based on a membrane potential formed across a lipid bilayer by different ion concentrations on either side of the membrane. Membrane leakage will therefore lead to a disturbance in this potential and in turn the conductance of the lipid bilayer. These disturbances, or lack thereof, can give insight into the ability of the peptides to form pores or channels in the cell membrane.

The same model lipid membranes used in ^{31}P solid state NMR experiments were used in the electrophysiological experiments namely: POPC:cholesterol (7:3), POPE:POPG (1:3) and POPE:POPG (3:1). After lipid bilayer formation on the MECA chips and bilayer stability determination; the peptides were added. Optimal pore formation for tryptocidine C was observed at 20-50 nM, while no pore/channel formation was observed for bacitracin A up to concentrations of 5 µM (data not shown). Single-channel events were used to construct the current jump histograms illustrated in Figure 3.22. It is evident, from Figure 3.22 A and B,

that the interaction of tryptocidine C with the membranes resulted in pore formation leading to predominantly ~2 pA currents and a narrow distribution in both POPC:cholesterol (7:3) and POPE:POPG (1:3). However, when tryptocidine C was added to the lipid combination POPE:POPG (3:1) that mimics Gram-negative cellular membranes, a broad distribution of pore sizes were observed (Figure 3.22 C). Furthermore, the predominant pore size was also larger (> 3 pA) than that observed for both the eukaryotic cellular membrane model (POPC:cholesterol (7:3)) and the Gram-positive cellular membrane model (POPE:POPG (1:3)).

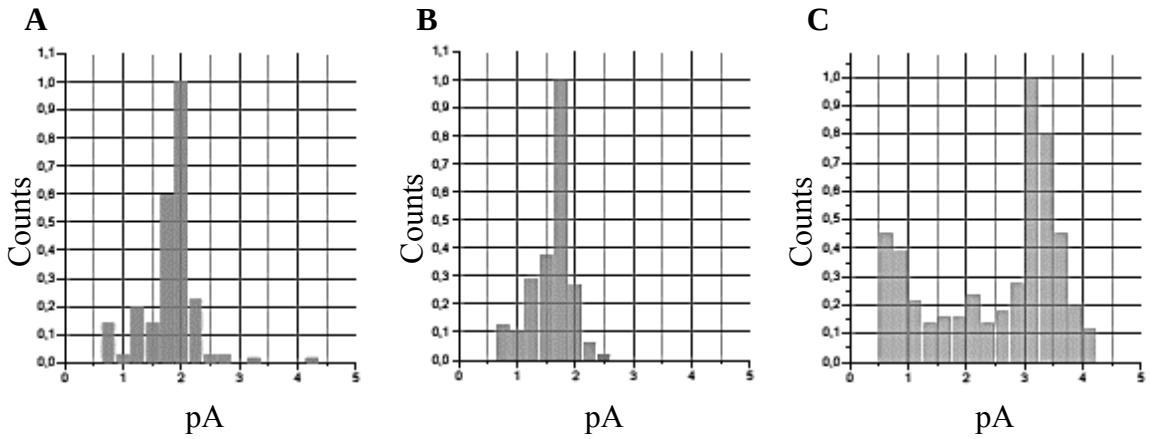


Figure 3.22. Single-channel current amplitude histograms for tryptocidine C in (A) POPC:cholesterol (7:3), (B) POPE:POPG (1:3) and (C) POPE:POPG (3:1) membranes. Counts refers to the number of times a pore with a specific amplitude was observed and is indicated as a fraction of the maximum count.

Both electrophysiological and ^{31}P solid state NMR experiments on lipid bilayers have confirmed the previous hypothesis that tryptocidine C exerts its antimicrobial activity via a membrane lytic or disruptive mode of action. Furthermore, narrow distribution of lipid bilayer pore sizes when tryptocidine C was added to POPC:cholesterol (7:3) and POPE:POPG (1:3) might be indicative of defined pore formation. The ^{31}P spectra of these peptide/lipid combinations indicated both disorientation of phospholipid head groups, as well as phase transition shifts by the peptides. However, a much greater effect on lipid bilayer orientation was observed as compared to the ^{31}P spectra of POPE:POPG (3:1) in the presence

of tryptocidine C. Furthermore, it was observed from electrophysiological experiments that tryptocidine C resulted in a broader distribution and generally larger pore/channel sizes when added to POPE:POPG (3:1). This result shows an indirect correlation with the lower activity that the peptides from the tyrocidine group, including the tryptocidines, have on Gram-negative bacteria [19]. This indicates that lipopolysaccharides may be preventing tryptocidines from eliciting antimicrobial activity against Gram-negative bacteria.

It is hypothesised that tryptocidine C orientates vertically across the lipid bilayers POPC:cholesterol (7:3) and POPE:POPG (1:3), thus resulting in some disorientation of phospholipid head groups and more defined pores. In contrast, tryptocidine C may insert parallel between the lipid layers of POPE:POPG (3:1) and therefore result in less phospholipid head group orientation disturbance and the formation of undefined random membrane failure, as was found for the analogous gramicidin S [4]. Further studies, however, are still needed to elucidate the exact orientation and interactions of tryptocidine C with the various lipid bilayers. The non-membrane active bacitracin A, as expected, did not result in any pore/channel formation or membrane failure when added to the lipid bilayers. Furthermore, bacitracin A displayed no haemolytic activity (section 3.4.4) yet interactions with the eukaryotic membrane equivalent (POPC:cholesterol) were observed, indicating that the lipid interactions observed by ^{31}P solid state NMR might only be to assist in the transportation of bacitracin to its subcellular target or to aid in the primary mode of action. Future studies will thus be focused on elucidating the role of the interaction between phospholipids and bacitracin A in its activity.

3.5 Conclusion

The antimicrobial compounds produced by the two bacterial isolates *B. licheniformis* LB.5 and *Br. laterosporus* LB.4 were isolated, purified and identified. *B. licheniformis* LB.5 was

convincingly shown to produce the antimicrobial peptide bacitracin A as previously hypothesised (Chapter 2) and was purified to a > 95 % purity. *Br. laterosporus* LB.4 was shown to produce two antimicrobial peptides with a M_r of 1222.6403 and 1273.6167 respectively, therefore, disproving our previous hypothesis that the isolate produces either BT1569 and BT1583 or bogorol A and bogorol B. No previously described antimicrobial agents with similar M_r values have been found after an extensive literature search, thus indicating that the discovered antimicrobial peptides may be novel. However, further studies are needed to ascertain the amino acid sequences and primary structures of these peptides and confirm novelty. Both of these peptides were purified to > 95% purity and preliminarily named, LB.4-1223 and LB.4-1273. The antimicrobial peptide tryptocidine C was also purified (> 95% purity) from the bacterium, *Br. parabrevis*, for the use as a control and proof of concept in purification, identification, bio-activity and biophysical studies.

Antimicrobial and haemolytic activity studies indicated that all of these peptides had activity towards *M. luteus* and erythrocytes, with the exception of bacitracin A that did not display any significant haemolytic activity. Biophysical studies on tryptocidine C and bacitracin A revealed their ability to interact with the cellular membranes of both eukaryotes and prokaryotes. Tryptocidine C resulted in defined membrane pores, supporting previous hypotheses of its ability to disrupt the cellular membrane of target organisms, as well as it lytic toxicity. In contrast bacitracin A did not display any pore formation indicating that cellular membrane interactions may only be to facilitate its transport to its intracellular target. These observations still need to be further investigated to confirm this hypothesis and to determine mechanism of interaction and the resulting response.

This study together with the results of the study described in Chapter 2 demonstrated the validity of the methods used for the isolation, purification and characterisation of antimicrobial peptides from environmental samples. In Chapter 4 we will describe the

isolation and preliminary identification of antimicrobial agents from an environmental soil sample using methods described in Chapter 2.

3.6 References

1. Marr, A. K., Gooderham, W. J. and Hancock, R. E. W. (2006) Antibacterial peptides for therapeutic use: obstacles and realistic outlook. Curr. Opin. Pharmacol. **6**, 468-472
2. Brogden, K. A. (2005) Antimicrobial peptides: pore formers or metabolic inhibitors in bacteria? Nat. Rev. Microbiol. **3**, 238-250
3. Leussa, A. N. (2014) Characterisation of small cyclic peptides with antimalarial and antilisterial activity. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/86161
4. Nguyen, L. T., Haney, E. F. and Vogel, H. J. (2011) The expanding scope of antimicrobial peptide structures and their modes of action. Trends Biotechnol. **29**, 464-472
5. Jenssen, H., Hamill, P. and Hancock, R. E. W. (2006) Peptide antimicrobial agents. Clin. Microbiol. Rev. **19**, 491-511
6. Ehrenstein, G. and Lecar, H. (1977) Electrically gated ionic channels in lipid bilayers. Q. Rev. Biophys. **10**, 1-34
7. Pouny, Y., Rapaport, D., Mor, A., Nicolas, P. and Shai, Y. (1992) Interaction of antimicrobial dermaseptin and its fluorescently labeled analogs with phospholipid membranes. Biochemistry. **31**, 12416-12423
8. Matsuzaki, K., Murase, O., Fujii, N. and Miyajima, K. (1996) An antimicrobial peptide, magainin 2, induced rapid flip-flop of phospholipids coupled with pore formation and peptide translocation. Biochemistry. **35**, 11361-11368
9. Bechinger, B. and Lohner, K. (2006) Detergent-like actions of linear amphipathic cationic antimicrobial peptides. Biochim. Biophys. Acta. **1758**, 1529-1539
10. Yeaman, M. R. and Yount, N. Y. (2003) Mechanisms of antimicrobial peptide action and resistance. Pharmacol. Rev. **55**, 27-55
11. Cotter, P. D., Ross, R. P. and Hill, C. (2013) Bacteriocins - a viable alternative to antibiotics? Nat. Rev. Microbiol. **11**, 95-105
12. Govaerts, C., Li, C., Orwa, J., Van Schepdael, A., Adams, E., Roets, E. and Hoogmartens, J. (2003) Sequencing of bacitracin A and related minor components by liquid chromatography/electrospray ionization ion trap tandem mass spectrometry. Rapid Commun. Mass Spectrom. **17**, 1366-1379
13. Werner, A. H. and Russell, A. D. (1999) Mupirocin, fusidic acid and bacitracin: activity, action and clinical uses of three topical antibiotics. Vet. Dermatol. **10**, 225-240
14. Stone, K. J. and Strominger, J. L. (1971) Mechanism of action of bacitracin: Complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. U. S. A. **68**, 3223-3227
15. Storm, D. R. and Strominger, J. L. (1974) Binding of bacitracin to cells and protoplasts of *Micrococcus lysodeikticus*. J. Biol. Chem. **249**, 1823-1827

16. Wu, X., Ballard, J. and Jiang, Y. W. (2005) Structure and biosynthesis of the BT peptide antibiotic from *Brevibacillus texasporus*. Appl. Environ. Microbiol. **71**, 8519-8530

17. Barsby, T., Warabi, K., Sørensen, D., Zimmerman, W. T., Kelly, M. T. and Andersen, R. J. (2006) The bogorol family of antibiotics: template-based structure elucidation and a new approach to positioning enantiomeric pairs of amino acids. J. Org. Chem. **71**, 6031-6037

18. Tang, X., Thibault, P. and Boyd, R. K. (1992) Characterisation of the tyrocidine and gramicidin fractions of the tyrothricin complex from *Bacillus brevis* using liquid chromatography and mass spectrometry. Int. J. Mass Spectrom. Ion Processes. **122**, 153-179

19. Dubos, R. J. (1939) Studies on a bactericidal agent extracted from a soil *Bacillus*: I. preparation of the agent. its activity *in vitro*. J. Exp. Med. **70**, 1-10

20. Spathelf, B. M. and Rautenbach, M. (2009) Anti-listerial activity and structure-activity relationships of the six major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Bioorg. Med. Chem. **17**, 5541-5548

21. Troskie, A. M. (2014) Tyrocidines, cyclic decapeptides produced by soil bacilli, as potent inhibitors of fungal pathogens. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/86162

22. Rautenbach, M., Vlok, N. M., Stander, M. and Hoppe, H. C. (2007) Inhibition of malaria parasite blood stages by tyrocidines, membrane-active cyclic peptide antibiotics from *Bacillus brevis*. Biochim. Biophys. Acta. **1768**, 1488-1497

23. Spathelf, B. M. (2010) Qualitative structure-actvity relationships of the major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/4001

24. Aranda, F. J. and de Kruijff, B. (1988) Interrelationships between tyrocidine and gramicidin A′ in their interaction with phospholipids in model membranes. Biochim. Biophys. Acta. **937**, 195-203

25. Lewis, J. C., Dimick, K. P. and Feustel, I. C. (1945) Production of tyrothricin in cultures of *Bacillus brevis*. Ind. Eng. Chem. **37**, 996-1004

26. Vosloo, J. A., Stander, M. A., Leussa, A. N., Spathelf, B. M. and Rautenbach, M. (2013) Manipulation of the tyrothricin production profile of *Bacillus aneurinolyticus*. Microbiology. **159**, 2200-2211

27. Salomon, R. A. and Farias, R. N. (1992) Microcin 25, a novel antimicrobial peptide produced by *Escherichia coli*. J. Bacteriol. **174**, 7428-7435

28. Du Toit, E. A. and Rautenbach, M. (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J. Microbiol. Methods. **42**, 159-165

29. Rasband, W. S. (1997-2015) ImageJ. U. S. National Institutes of Health, Bethesda, Maryland, USA. http://imagej.nih.gov/ij/

30. Rautenbach, M., Gerstner, G. D., Vlok, N. M., Kulenkampff, J. and Westerhoff, H. V. (2006) Analyses of dose-response curves to compare the antimicrobial activity of

model cationic α-helical peptides highlights the necessity for a minimum of two activity parameters. Anal. Biochem. **350**, 81-90

31. Johnson, B. A., Anker, H. and Meleney, F. L. (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science. **102**, 376-377

32. Konz, D., Klens, A., Schörgendorfer, K. and Marahiel, M. A. (1997) The bacitracin biosynthesis operon of *Bacillus licheniformis* ATCC 10716: molecular characterization of three multi-modular peptide synthetases. Chem. Biol. **4**, 927-937

33. Eyéghé-Bickong, H. A. (2011) Role of surfactin from *Bacillus subtilis* in protection against antimicrobial peptides produced by *Bacillus* species. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/6773

34. Vosloo, J. A. (2016) Optimised bacterial production and characterisation of natural antimicrobial peptides with potential application in agriculture. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/98411

35. Rammelkamp, C. H. and Weinstein, L. (1941) Hemolytic effect of tyrothricin. Exp. Biol. Med. **48**, 211-214

36. Epand, R. M. and Vogel, H. J. (1999) Diversity of antimicrobial peptides and their mechanisms of action. Biochim. Biophys. Acta. **1462**, 11-28

37. Bechinger, B. and Sizun, C. (2003) Alignment and structural analysis of membrane polypeptides by ^{15}N and ^{31}P solid-state NMR spectroscopy. Concepts Magn. Reson. **18A**, 130-145

38. Strandberg, E. and Ulrich, A. S. (2004) NMR methods for studying membrane-active antimicrobial peptides. Concepts Magn. Reson. **23**, 89-120

Chapter 4
Isolation and identification of antimicrobial producing bacteria from a soil environment

4.1 Introduction

The soil biome houses a vast diversity of bacterial species which surpasses that of any other environment [1]. Soil environments are in most part static and devoid of profuse nutrient mixing. The static environment in turn results in limited nutrients to soil microorganisms as the nutrients do not get frequently replaced after being metabolised. Therefore soil microorganisms have developed several methods to outcompete neighbouring microorganisms for the limited supply of nutrients, one of which is the production of antimicrobial peptides [2-4].

Since the early 1940's, after the discovery of the first antimicrobial peptide complex, tyrothricin, hundreds of antimicrobial peptides have been identified from soil bacteria, many of which have important functions as clinical antibiotics and food preservatives [5-8]. Although soil environments have been abundantly screened for new antimicrobial peptides, only a small fraction of the earth's soil microbiome have been mined as of yet [9]. Furthermore, only 1% of soil microorganisms, as shown by genomic studies, have been cultured using current laboratory techniques [10]. The remaining 99% of "microbial dark matter" might still contain valuable novel antimicrobial peptides [11,12]. Therefore, the historical success and unexploited potential of the soil biome makes it an invaluable source of novel antimicrobial peptides.

In this study we isolated various soil bacteria from a garden soil sample taken near the Biochemistry Department at University of Stellenbosch. The isolates were screened for their antimicrobial and haemolytic activity. The molecular mass (M_r) of small molecules (between

800 and 3000) produced by the antimicrobial active isolates were determined and compared to antimicrobial agents in literature to establish whether isolates produced novel antimicrobial peptides, so as to be considered in future purification, identification and characterisation studies.

4.2 Materials

4.2.1 Bacterial strains

American Type Culture Collection (Manassas, VA, USA) and National Collection of Type Cultures (Proton Down, Salisbury, United Kingdom) supplied cultures of *Brevibacillus parabrevis* ATCC 10068 and *Micrococcus luteus* NCTC 8340 respectively. Bacterial isolates where obtained from a soil sample in a courtyard next to the JC Smuts Building at the University of Stellenbosch in Stellenbosch, South Africa.

4.2.2 Research materials

The materials used in this study have been previously described in Chapter 2, section 2.2.2.

4.3 Methods

4.3.1 Isolation of bacteria from soil

A soil sample was collected by removing the top 2 cm of the soil sample area, after which a sterile spatula was used to transfer soil until a depth of 15 cm into sterile 50 mL polypropylene centrifuge tubes. A sterile spatula was used to further transfer soil from the sample into two separate sterile 1.5 mL microfuge tubes. Sterile analytical grade water (1.0 mL) was added to the soil samples and then slowly vortexed to suspend soil bacteria. One tube was left at room temperature for 3 minutes to allow any soil particulates to settle out. The second tube was heated to 80°C on a heating block for 3 minutes to allow soil particulates to settle out. The different incubation conditions were included to ensure the

isolation of diverse bacterial species, with the 80°C heating step aiding in selection of sporulating organisms [13]. The suspensions were then subjected to a 10 times serial dilution in sterile analytical grade water to a dilution factor of 10^{-7}. Each serial dilution was spread onto two plates of LB, NB, and TSB agar (1.5% agar) growth media. To ensure the growth of a large diversity of bacterial species, one plate of each type of growth medium was incubated at 37°C whereas the other was incubated at 25°C. All plates where inspected every 24 hours up until 72 hours and photos taken with a ChromaDoc-It TLC imaging system (UVP, California, USA) connected to a Canon Eos Rebel T3 digital camera (Canon, Tokyo, Japan) to monitor colony growth. After 72 hours, colonies from all plates where inspected for difference in colony morphology. Morphologically different colonies where then selected and streaked onto their respective mediums, initially isolated from, and incubated at 25°C or 37°C, depending on the initial isolation condition, for 48 hours to obtain pure colonies. Colonies were named according to the growth media, whether they were subjected to 80°C or 25°C and the initial incubation temperature. A pure colony was then selected and streaked out on both LB agar and TSB agar and stored at 4°C for less than a week. Pure colonies from the 4°C stocks where streaked out onto both LB agar and TSB agar and incubated for 48 hours at 25°C or 37°C, depending on the initial isolation condition, before antimicrobial and haemolytic activity analyses were performed as described in section 4.3.2 and section 4.3.3 respectively.

4.3.2 Identification of antimicrobial producing isolates

Determination of antimicrobial activity of the soil isolates was done by the simultaneous antagonism spot-on-lawn method adapted from Tagg *et al*. [14] and Du Toit & Rautenbach [15] as described in detail in section 2.3.2 of Chapter 2. Briefly, pure soil isolate colonies were spotted with a sterile pipette tip onto a lawn of *M. luteus* NCTC 8340. The clear inhibition zones produced by the soil isolates where monitored at 24 and 48 hours of

incubation at 25°C or 37°C, depending on the isolation conditions of the specific soil isolates (see section 4.3.1). Photos were taken at 24 and 72 hours with the imaging system described in section 4.3.1. The inhibition zone size produced by isolates was then compared to that of the positive control, *Br. parabrevis*, which produce the tyrocidine antimicrobial peptide group. Colony spots of *M. luteus* was used as a negative control.

4.3.3 Preliminary determination of isolate haemolytic toxicity

Soil isolates were subjected to blood plate analyses as described in section 2.3.3 of Chapter 2 to determine if they possess haemolytic activity. Plates containing erythrocyte-enriched TSB agar media where spotted with the soil isolates using sterile pipette tips. The plates where then incubated at either 25°C or 37°C according to isolation conditions (see section 4.3.1). The clear haemolytic zones of the soil isolates compared to that of *Br. parabrevis*, which produces highly haemolytic peptides from the tyrocidine group of antimicrobial peptides, at 24 and 48 hours. *M. luteus* was used as a negative control. The imaging system described in section 4.3.1 was used to take photos of the plates at 24 and 48 hours.

4.3.4 ESMS analysis of the selected antimicrobial producing isolates.

Soil isolate colonies that displayed antimicrobial activity were spotted into a 500 µL TSB agar containing 1.5 mL microfuge tube. After incubation at 37°C for 72 hours, the cultures were extracted with 50% acetonitrile and the extracts subjected to electrospray mass spectrometry (ESMS) analysis as described previously in section 2.3.5 of Chapter 2. The resulting spectra were then analysed with Waters MassLynx V4.1 software (Milford, USA) and compared to that of the negative control (sterile TSB agar) and the positive control (*Br. parabrevis*) to identify soil isolates that possibly produces antimicrobial peptides in the M_r range of 800 to 3000. ESMS spectra were charge deconvoluted by the MaxEnt algorithm in the MassLynx V4.1 software to obtain experimental monoisotopic M_r values where specified.

4.3.5 Bacterial identification

Matrix assisted laser desorption ionization mass spectrometry (MALDI-MS) biotyping and 16S rRNA gene sequencing, as previously described in section 2.3.6 of Chapter 2, were used to identify the bacterial species suspected of producing antimicrobial peptides.

4.4 Results and discussion

4.4.1 The isolation of antimicrobial producing bacteria from soil

A soil sample was collected near the Department of Biochemistry, University of Stellenbosch, Stellenbosch, South Africa. The surrounding soil is mostly in the shade of nearby trees and generally receives minimal sunlight. It was also observed to be generally moist with groundcover plants. The sample was serially diluted in analytical grade water and spread onto TSB, LB and NB agar to facilitate the growth of fastidious and non-fastidious bacterial species. Single colonies where selected based on morphological differences from plates where bacterial colonies did not overlap and could be selected with ease.

The selection resulted in 509 colonies displaying different colony morphologies, as determined visually. Antimicrobial spot-on-lawn analysis of the isolates, using *M. luteus* as an indicator organism, revealed that only 15 of the 509 isolates exhibited antimicrobial activity, as indicated by clear inhibition zones surrounding isolate colonies. However, 74 isolates where determined to have haemolytic activity using blood plate analysis. Only isolates that displayed antimicrobial activity towards *M. luteus* were assessed in further studies.

The large numbers and variety of bacteria that were isolated from soil made the selection of colonies with different morphologies difficult, thus increasing the odds of selecting a specific type of colony morphology more than once (Figure 4.1 and Table 4.1). Furthermore, the morphology of some bacteria changed after they were selected and streaked onto LB and

TSB agar to obtain pure colonies. This might be due to the depth of the media bed and slight changes in media composition [16]. Most of the soil isolates that displayed antimicrobial activity after 48h differ in their colony morphologies (Table 4.1). However, it can be seen from Table 4.1 that there are active isolates displaying similar colony morphologies which include: N2537-3 and N2537-6, N2537-15 and N2537-40, L2525-30 and L2525-31. Colony morphology is not an absolute discriminating technique for identifying different bacterial strains [17]. Therefore, it is possible that bacterial colonies with similar morphologies might be different strains and thus may produce different antimicrobial agents. In contrast, a single strain of bacteria may have different colony morphologies depending on environmental conditions [16,18]. Thus, to truly determine whether different isolates displaying activity towards *M. luteus* are different strains of bacteria and/or produce different antimicrobial agents, further investigation with alternative assays and selection methodologies are needed. However, screening based on colony morphological differences are rapid and undemanding and was therefore sufficient in this study as initial screening methodology.

Figure 4.1. Example of a LB agar plate displaying the growth of different soil bacterial colonies

Table 4.1. Morphological characteristics of soil isolates displaying antimicrobial activity towards *M. luteus*

Isolate [a]	Shape	Margin	Elevation	Size	Texture	Appearance	Pigmentation
L2525-30[1]	Irregular	Curled	Convex	Medium	Smooth	Glistening	Clear/White
L2525-31[1]	Irregular	Curled	Convex	Medium	Smooth	Glistening	Clear/White
L2537-30	Irregular	Lobate	Convex	Small	Rough	Dull	White/Brown
L8025-22	Circular	Undulate	Raised	Medium	Smooth	Dull	Tan
N2525-7	Circular	Entire	Flat	Small	Smooth	Dull	Clear
N2537-3[2]	Circular	Entire	Raised	Large	Smooth	Dull	White/Cream
N2537-6[2]	Circular	Entire	Raised	Large	Smooth	Dull	White/Cream
N2537-15[3]	Irregular	Lobate	Umbonate	Small	Rough	Dull	White/Brown
N2537-40[3]	Irregular	Lobate	Umbonate	Small	Rough	Dull	White/Brown
N2537-48	Irregular	Curled	Pulvinate	Small	Smooth	Dull	Cream
N8025-5	Circular	Entire	Flat	Medium	Smooth	Dull	Tan
N8037-14	Circular	Curled	Raised	Medium	Rough	Glistening	Cream
T2525-6	Irregular	Undulate	Raised	Small	Rough	Dull	Cream
T2537-10	Irregular	Curled	Convex	Small	Rough	Dull	White/Brown
T8037-7	Circular	Entire	Flat	Large	Smooth	Dull	Tan

[a] Isolates displaying similar colony morphologies are indicated by [1-3] respectively.

It was found that seven of the 15 antimicrobial active isolates had higher antimicrobial activity towards *M. luteus* than *Br. parabrevis* (Table 4.2). Antimicrobial activity towards *M. luteus* of eight of the antimicrobially active isolates was comparable to that of *Br. parabrevis* (Table 4.2). Haemolytic activity analysis revealed that three of the antimicrobial active soil isolates displayed higher haemolytic activity than *Br. parabrevis* (Table 4.3). Little to no haemolytic activity was observed for the remaining antimicrobially active isolates (Table 4.3). When comparing antimicrobial and haemolytic activity, it can be seen that several of the antimicrobial active soil isolates displayed similar antimicrobial/haemolytic activity combinations (Table 4.2 and Table 4.3). Taken together with morphological characteristics, it

was found that only the L2525-30 and L2525-31 isolates displayed similar morphologies and antimicrobial/haemolytic activity combinations. Furthermore, antimicrobial soil isolates with similar morphologies (N2537-3 and N2537-6, N2537-15 and N2537-40) had different antimicrobial/haemolytic activity combinations. Therefore, indicating that screening based on colony morphology characteristics is not an accurate means of differentiating between different bacterial species or strains.

Table 4.2. Antimicrobial activity of soil isolates towards *M. luteus*.

Isolate	Growth [a]		Antimicrobial activity [b]	
	24h	48h	24h	48h
L2525-30	+	++	+	++
L2525-31	+	++	+	++
L2537-30	+	++	-	+
L8025-22	+	++	++	++
N2525-7	+	+	±	+
N2537-3	++	++	±	++
N2537-6	+++	+++	+	+++
N2537-15	+	++	-	+
N2537-40	++	++	±	+
N2537-48	+	++	±	+
N8025-5	+	+	++	++
N8037-14	++	++	±	++
T2525-6	+	++	+	+
T2537-10	+	++	-	+
T8037-7	++	++	-	+

[a] The colony size (growth) of isolates were compared to that of *Br. parabrevis* after 24 and 48 hours. The + sign is indicative of similar colony size whereas ++ or +++ refers to a larger colony size.

[b] The size of clear zones, due to inhibition of *M. luteus* growth, produced by isolates were compared to the inhibition zone formed by *Br. parabrevis* after 24 and 48 hours. The + sign is used to indicate inhibition zones of similar size whereas ++ and +++ refers to larger inhibition zones and ± refers to smaller inhibition zone. The - sign refers to the absence of an inhibition zone.

Table 4.3. Haemolytic activity of soil isolates displaying antimicrobial activity towards *M. luteus*

Isolate	Growth [a]		Haemolytic activity [b]	
	24h	48h	24h	48h
L2525-30	+	++	-	-
L2525-31	+	++	-	-
L2537-30	+	++	-	±
L8025-22	+	+	-	-
N2525-7	-	±	-	-
N2537-15	+	+	-	-
N2537-3	++	+++	++	++
N2537-40	+	+	-	±
N2537-48	-	-	-	-
N2537-6	++	+++	++	++
N8025-5	+	+	-	na
N8037-14	++	+++	+	++
T2525-6	-	±	-	-
T2537-10	+	+	-	±
T8037-7	+	+	-	±

[a] The colony size (growth) of isolates were compared to that of *Br. parabrevis* after 24 and 48 hours. Colonies of similar size are indicated by +, whereas ++ or +++ refers to a larger a colony size and ± a smaller colony size. No visible growth is indicated by the - sign.
[b] Clear haemolytic zones sizes were compared to the haemolytic zone size produced by *Br. parabrevis* after 24 and 48 hours. The + sign is used to indicate haemolytic zones of similar size, whereas ++ and ± refers to a larger haemolytic zone and smaller haemolytic zone respectively. The - sign is indicative of the absence of a haemolytic zone. A colony that was overgrown by neighbouring isolates, making zone identification improbable, is indicated with "na".

4.4.2 ESMS analysis of low M_r compounds produced by antimicrobially active soil isolates

The low M_r compound profile of soil isolates that displayed antimicrobial activity towards *M. luteus* where determined by a newly developed ESMS technique as described in Chapter 2. The selected soil isolates where subjected to direct organic extraction from TSB agar following 72 hours of incubation at 37°C. The resulting supernatants where then subjected to ESMS analysis. ESMS spectra of the soil isolates were subsequently compared to that of the sterile TSB agar (negative control) and *Br. parabrevis* (positive control) extracts. *Br. Parabrevis* produces the antimicrobial peptide complex tyrothricin, which comprises of a variety of tyrocidine analogues as well as some gramicidins [19]. Experimental success was

confirmed when tyrocidine analogues, for example tyrocidine A tyrocidine B and tyrocidine C, could be detected with the ESMS method.

The ESMS spectra of both the negative control and the positive control indicated a large amount of background noise between the M_r values from 300 to 800 (Figure 4.2 A and B). However, from Figure 4.2 B it is clear that tyrocidine analogues, as well as gramicidins produced by *Br. parabrevis* could still be distinguished, validating the method. Tyrocidine analogues that were found to be produced under the culturing conditions include tyrocidine A (M_r = 1296.6501) and tyrocidine B (M_r = 1308.6570), together with their sodium adducts (Figure 4.2 C). The extensive background noise below M_r 800 made distinguishing the low M_r profiles of smaller antibiotic compounds in samples unlikely (Figure 4.2 A and B). In the negative control (extract from sterile TSB agar) several peaks with significant intensity are observed between the M_r values of 1120 and 1165 (Figure 4.2 A). These peaks, however, were absent or had insignificant ESMS signal intensities when bacterial cultures were present and were therefore not considered as background noise. Only compounds with M_r values between 800 and 3000, which are likely to be antimicrobial peptides, were considered in the ESMS analyses of soil isolates.

ESMS analysis revealed five antimicrobial active soil isolates that produced compounds with a M_r greater than 800 namely: T8037-7, N8037-14, N2537-15, N2537-40 and L2537-30. No compounds with significant signal intensity between the M_r values of 800 and 3000 were observed using the ESMS method for the remaining 10 isolates (Addendum A). This might be due to either the absence of antimicrobial production during the analysis or the production of antimicrobial compounds with a M_r below 800 making identification improbable due to high background noise. Thus, further purification steps will be necessary to aid in the removal of the current background noise and the identification of possible antimicrobial compounds with a M_r below 800.

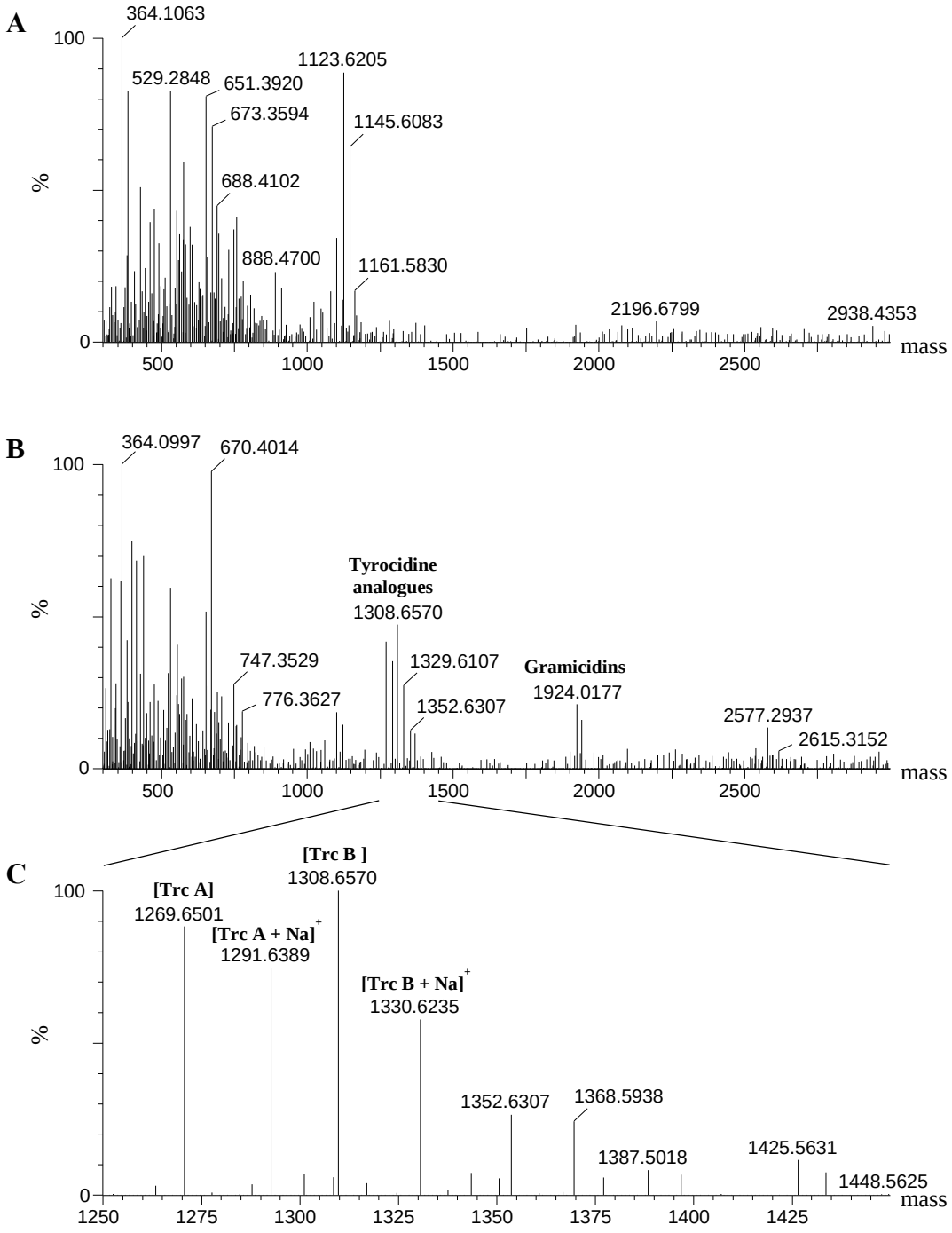


Figure 4.2. ESMS analysis of sterile TSB media and *Br. parabrevis*. MaxEnt analysis of the ESMS spectra obtained from sterile TSB agar display high levels of background noise between the M_r values of 300 and 800 (A). Peaks observed between the M_r values of 1120 and 1165 from sterile TSB agar were not observed when cultures were present and were therefore not considered background noise. MaxEnt analysis of the ESMS spectra from *Br. parabrevis* between the M_r of 300 and 3000 show the presence of tyrocidine analogues and gramicidins (B). The ESMS spectra after MaxEnt analysis within the M_r range of 1250 and 1450 display the presence of tyrocidine A (Trc A) and tyrocidine B (Trc B) together with their sodium adducts (C).

The soil isolate T8037-7 was found to produce a compound with a *Mr* of 1057.6705, which was the only compound observed within the M_r range of 800 to 3000 with significant ESMS signal intensity (Figure 4.3). The determined M_r of 1057.6705 was found to correlate with the M_r of the antimicrobial peptide from the iturin group of peptides, iturin C2 with a M_r of 1057.5444, as indicated by Table 4.4 [20]. Iturin is a group of cyclic lipopeptides non-ribosomally produced by strains of *Bacillus subtilis* that contain varying acyl chains linked to a cyclic peptide moiety [21]. Iturin C2 belongs to the subgroup iturin C, which contains several analogues differing in acyl chain length [20]. Iturin C has been shown to not possess antimicrobial or haemolytic activity, contradicting observations in section 4.3.1 [22,23].

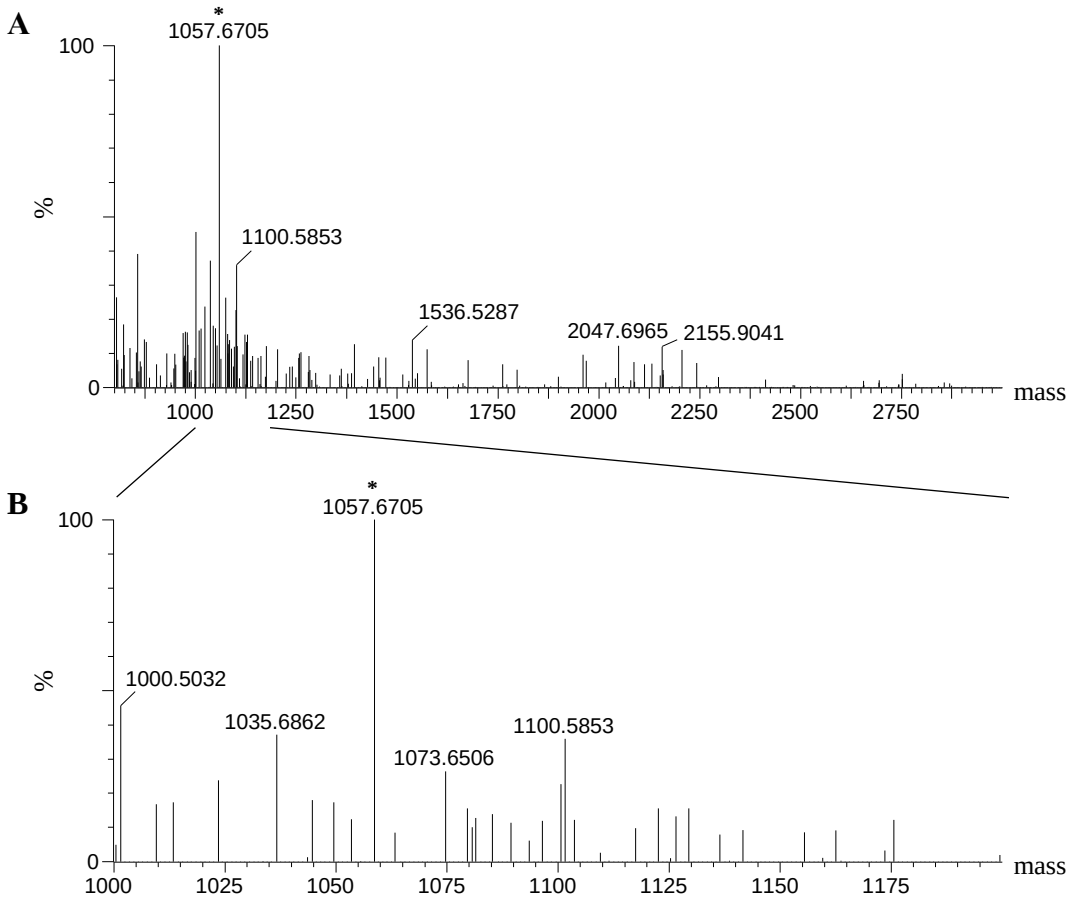


Figure 4.3. ESMS analysis of the soil isolate, T8037-7, displaying spectra between the M_r values of 800 to 3000 (A) and 1000 to 1200 (B) following MaxEnt analysis. The presence of the compound suspected to be responsible for antimicrobial activity is indicated by *.

Co-produced iturin A, however, has been shown to possess both antimicrobial and haemolytic activity [23-26]. Iturin A differs from iturin C by the substitution of the aspartyl residue for an asparaginyl residue [22,27]. During iturin production *B. subtilis* naturally produced a complex op lipopeptides with a variety of differing acyl chain lengths (14 difference in M_r) visible on ESMS spectra, which is not the case for this isolate [28]. Furthermore, the ESMS on the T8037-7 extract was done at high resolution (< 10 ppm mass error) and the difference between the M_r of the major extracted compound and iturin C2 is 0.1261 Da (or 126 ppm), which does not point to a single amino acid mutation (Table 4.4). Thus the absence of molecular species with methyl group differences, contradicting bio-activity and large mass error led us to conclude that it is improbable that the T8037-7 isolate produces itrurin C2. Further analysis following purification is therefore necessary to identify the antimicrobial compound(s) produced by the T8037-7 isolate.

The soil isolate, N8037-14, was observed to produce a major compound with a M_r of 1353.8691, which was detected together with its sodium adduct (M_r of 1375.8466), as indicated by the significant ESMS signal intensity within the M_r range of 800 to 3000 (Figure 4.4). As indicated in Table 4.4 the M_r of 1353.8691 was found to correspond fairly closely with only the M_r of 1353.8007 of the biosurfactant peptide, arthrofactin [29]. The ESMS on the N8037-14 isolate extract was also done at high resolution (< 10 ppm mass error). The M_r difference between the major extracted compound and athrofactin is 0.0684 (or 68 ppm), which does not point to a single amino acid mutation. This makes athrofactin an unlikely candidate for the antimicrobial activity of the N8037-14 isolate (Table 4.4). The soil isolate, N8037-14, might therefore produce a novel antimicrobial compound with a M_r of 1353.8691. A second compound with significant ESMS signal intensity was observed to be produce by the N8037-14 isolate with a M_r value of 1583.0824 (Figure 4.4). From literature it was found that the M_r of the compound closely matched that of the antimicrobial peptides BT1583 (M_r =

1583.1154) and bogorol A (M_r = 1583.0790) displayed in Table 4.4 [30,31]. BT1583 peptide differed from the compound with a M_r value of 1583.0824 by 0.0330 Da (or 33 ppm) which is outside the mass error range and does not correlate to a single amino acid mutation (Table 4.4). It is therefore unlikely that N8037-14 produces BT1583 peptide. The compound with the M_r of 1583.0824 is more likely to be bogorol A, due to the low mass error of 3 ppm.

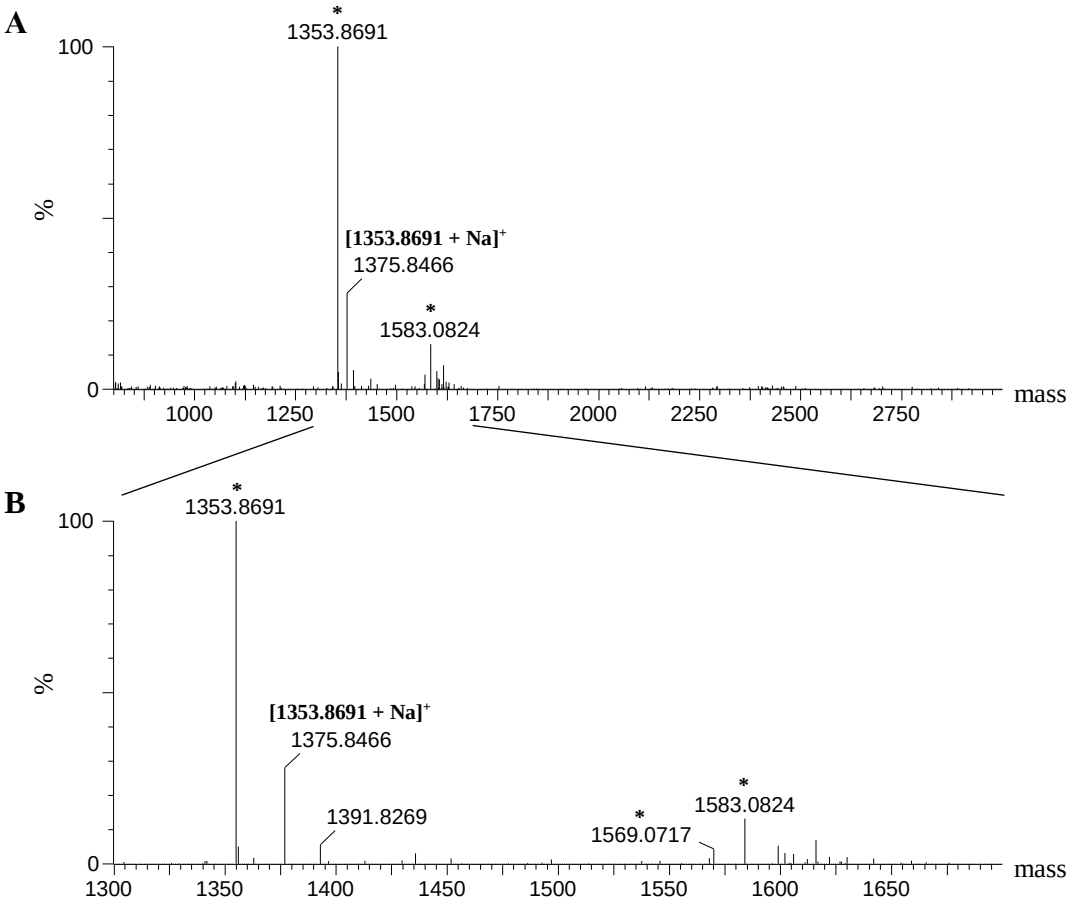


Figure 4.4. ESMS analysis of the soil isolate, N8037-14, displaying spectra between the M_r values of 800 to 3000 (A) and 1300 to 1700 (B) following MaxEnt analysis. Compounds suspected of having antimicrobial activity are indicated with by *.

Although the ESMS signal intensity of the compound with a M_r of 1569.0717 was low as seen in Figure 4.4 B, it was found that the M_r of the compound was similar to that of the antimicrobial peptides BT1569 (M_r = 1569.0097) and bogorol B (M_r = 1569.0633) as shown in Table 4.4 [30,31]. BT1569 differed from the compound with a M_r value of 1569.0717 by 0.0620 (or 62 ppm) which again did not correlate to a single amino acid mutation and is

outside the mass error range. Therefore, the compound with a M_r of 1569.0717 is unlikely to be BT1569. The M_r difference of 8 ppm compared to bogorol B indicates that the compound at a M_r of 1569.0717 may be bogerol B. Both bogorol A and bogorol B together with several other peptides form part of a closely related group of co-produced antimicrobial peptides collectively referred to as bogorols [31]. Therefore the compounds with M_r of 1569.0717, despite its low ESMS signal intensity, and 1583.0824 were considered to be possible candidates for the antimicrobial activity of the soil isolate, N8037-14, due to the similar M_r values.

These results indicate that the N8037-14 isolate might produce a possibly novel antimicrobial (M_r = 1353.8691) and/or the bogorol A and bogorol B antimicrobial peptides, instead of athrofactin, BT1583 or BT1569 peptides. The compounds with M_r values of 1569.0717 and 1583.0824 also have similar M_r values as two compounds produced by the soil additive isolate, *Brevibacillus laterosporus* LB.4, discussed in Chapter 2 and Chapter 3. However, ESMS spectra of *Br. laterosporus* LB.4 did not display the presence of a compound with a M_r value of 1353.8691. Further investigations are thus needed to correctly establish and confirm the identity of the molecular species responsible for the antimicrobial characteristics of the N8037-14 isolate.

The low M_r profiles of N2537-15, N2537-40 and L2537-30 as determined by ESMS analysis were found to be similar (Figure 4.5 and Addendum A). Furthermore, colony morphology of the isolates N2537-15 and N2537-40 correlated and the morphology of the L2537-30 isolate colony only slightly varied in elevation (Table 4.1). This suggested that these isolates might be from the same bacterial species or strain. This observation again indicates that screening for different bacterial species or strains according to colony morphological characteristics is not the most accurate method for the selection of different bacterial species or strains from environmental samples. The soil isolates, N2537-15 and L2537-30, were not included in

further investigations and discussions due to the morphological and ESMS spectra similarities compared to the N2537-40 isolate, which displayed the highest ESMS signal intensities for each molecular species. Higher ESMS signal intensity may translate to higher antimicrobial compound production.

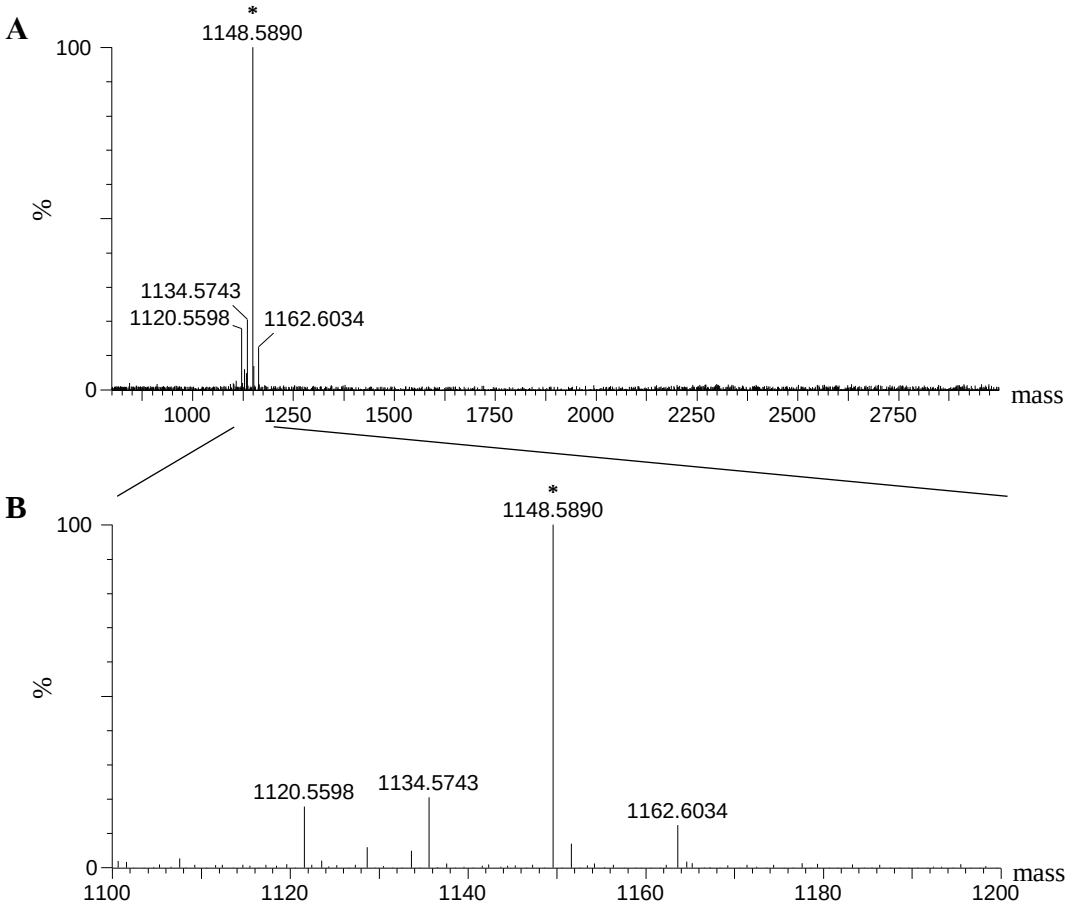


Figure 4.5. ESMS analysis of the soil isolate, N2537-40, displaying spectra between the M_r values of 800 to 3000 (A) and 1100 to 1200 (B) following MaxEnt analysis. The * refers to the main compound suspected to be antimicrobially active.

From the ESMS spectrum it was observed that the soil isolate, N2537-40, produced one major compound with a M_r of 1148.5890 (Figure 4.5). Several species displaying lower ESMS signal intensities were also detected which includes compounds with M_r values of 1120.5598, 1134.5743 and 1162.6034 (Figure 4.5 B). Compared to the major compound (M_r = 1148.5890) the compounds with M_r values of 1134.5743 and 1162.6034 differs by a M_r 14.01 whereas the compound with a M_r value of 1120.5598 differs by 28.03. The M_r

difference of 14.01 and 28.03 is indicative of a methyl group (CH_2) or ethyl group difference from the major compound respectively. Therefore, the N2537-40 isolate might produce a lipopeptide with a varying acyl chain. Alternatively it could be a peptide with analogues varying in either Leu or Val; Glu or Asp; Gln or Asn or lastly Lys or Orn residue(s). No naturally produced antimicrobial compounds could be found in literature that had M_r values similar to those observed to be produced by the N2537-40 isolate. This suggested the possibility of a novel antimicrobial compound, possibly a lipopeptide. Future investigations will therefore be focussed on purification, identification and characterisation of the antimicrobial compound produced by the N2537-40 isolate.

4.4.3 Bacterial identification

The soil isolates T8037-7, N8037-14 and N2537-40 were subjected to bacterial identification by both MALDI-MS biotyping and 16S rRNA gene sequencing. MALDI-MS biotyping identified the T8037-7 isolate to be *Bacillus subtilis* with a biotyper score of 2.141, indicative of a secure genus identification and probable species identification (Table 4.4). From 16S rRNA gene sequencing results, T8037-7 was determined to be either *Bacillus oryzicola* or *B. subtilis* both with 100% sequence identity (Table 4.4). The T8037-7 isolate was therefore securely identified to be from the genus *Bacillus*. Several *B. subtilis* strains naturally produce the group of related antimicrobial peptides referred to as iturins which includes iturin C2 [24,27]. From ESMS analysis (section 4.4.2) it was observed that the T8037-7 isolate produces a low M_r compound closely resembling the M_r of the antimicrobial peptide, iturin C2, but we observed conflicting bio-activity and an absence of a lipopeptide complex spectra, which is characteristic of iturin production (Figure 4.3, Table 4.3 and Table 4.4) [20,28]. No clear conclusion could be made regarding the species identity of the T8037-7 isolate. Further investigation is, therefore, still needed to securely identify the species of *Bacillus* which the T8037-7 isolate belongs to.

The soil isolate, N8037-14, was identified to be *Brevibacillus laterosporus* by 16S rRNA gene sequencing with a sequence identity of 99% (Table 4.4). No reliable identification was obtained by MALDI-MS biotyping (Table 4.4). From ESMS spectra, discussed in section 4.4.2, it was observed that the N8037-14 isolate produced two compounds with M_r values closely matching that of the antimicrobial peptides bogorol A and bogorol B. These peptides are naturally produced by a strain of *Br. laterosporus* (Table 4.4) [31]. It is therefore highly likely that the N8037-14 isolate is a strain of *Br. laterosporus*. ESMS spectra in section 4.4.2 also indicated that the N8037-14 isolate may produce compounds with M_r similar to that of BT1583, BT1569 and arthrofactin (Table 4.4). The difference in producing species as well as M_r differences that are outside the mass error range and also do not relate to single amino acid mutation, directed us to discard BT1583, BT1569 and arthrofactin as candidates for the antimicrobial activity of the N8037-14 isolate (Table 4.4). The presence of a compound with a M_r of 1353.8691, which has not yet been securely identified, necessitates further investigation by purification and identification of the antimicrobial compounds produced by the N8037-14 isolate. Future studies will also be focused at identifying the specific bacterial strain.

The N2537-40 isolate was identified as *Streptomyces badius* by MALDI-MS biotyping with a biotyper score of 1.967, which is indicative only of a probable genus identification (Table 4.4). 16S rRNA gene sequencing also identified the isolate N2537-40 to belong to the genus *Streptomyces* (Table 4.4). However, the 16S rRNA gene sequence displayed similar percentage sequence identities towards several *Streptomyces* species. The low M_r profile observed for the N2537-40 isolate (section 4.4.2) could not be used to aid in species identification due to the absence of compounds in literature with similar M_r values to that observed during ESMS analysis (Table 4.4). Therefore, further investigation is needed to securely identify species using different primer sets.

Table 4.4. Summary of the bacterial identification and low M_r profiles of the soil isolates: T8037-7, N8037-14 and N2537-40.

Isolate	Major compounds M_r [a]	MALDI-MS biotyping		16S rRNA gene sequensing		Similar peptides				M_r difference (ppm) [g]
		Identification	Biotyper score [b]	Identification	% Sequence identity [c]	Peptide [d]	M_r [e]	Producing organism [f]	Ref	
T8037-7	1057.6705	*Bacillus subtilis*	2.141	*Bacillus oryzicola or Bacillus subtilis*	100	iturin C2 *	1057.5444	*Bacillus subtilis*	[20]	126
N8037-14	1353.8691	No ID	1.549	*Brevibacillus laterosporus*	99	arthrofactin *	1353.8007	*Arthrobacter* sp.	[29]	68
	1583.0824					BT1583 *	1583.1154	*Brevibacillus texasporus*	[30]	33
						bogorol A	1583.0790	*Brevibacillus laterosporus*	[31]	3
	1569.0717					BT1569 *	1569.0097	*Brevibacillus texasporus*	[30]	62
						bogorol B	1569.0633	*Brevibacillus laterosporus*	[31]	8
N2537-40	1148.5890 1120.5598 1134.5743 1162.6034	*Streptomyces badius*	1.967	*Streptomyces* spp.	99	None found	-	-	-	-

[a] The experimental monoisotopic M_r values of compounds produced by the selected soil isolates with significant ESMS signal intensities.
[b] MALDI-MS biotyping scores reflect as follows: 2.300 - 3 .000 = highly probable species identification, 2.000-2.299 = secure genus identification and probable species identification, 1.700 – 1.999 = probable genus identification, 0.000 – 1.699 = no reliable identification.
[c] Percentage nucleotide sequence identity as determined by NCBI Genbank database blast.
[d] Antimicrobial peptides from literature with similar M_r values to those of compounds produced by the isolates. Unlikely candidates for the isolate's antimicrobial activity are indicated by *.
[e] The monoisotopic M_r values of peptides found from literature with similar M_r values as compounds produced by the selected soil isolates.
[f] The producing organisms of peptides from literature with similar M_r values to compounds produced by soil isolates
[g] The M_r difference as calculated by the subtraction of the M_r values from compounds produced by the selected isolates and similar antimicrobial peptides found in literature.

4.5 Conclusion

A soil sample collected near the Department of Biochemistry, University of Stellenbosch, South Africa yielded 507 morphologically different soil bacterium colonies. Three isolates; T8037-7, N8037-14 and N2537-40, were selected due to desirable antimicrobial activity and production of low M_r compounds. The T8037-7 isolate was shown to be a *Bacillus* sp. which produces a compound with a similar M_r as iturin C2. However, due to a large mass error in M_r values and dissimilarities towards characteristic iturin production profiles, it was concluded that the T8037-7 isolate does not produce iturin C2. Future investigations will include both species identification as well as the identification of the antimicrobial compound produced by the T8037-7 isolate. The N8037-14 isolate, belonging to the species *Br. laterosporus*, was hypothesised to produce either a novel antimicrobial agent and/or the antimicrobial peptides bogorol A and bogorol B. Further investigation will however need to be focused at identifying the antimicrobial agent(s) as a compound was observed that could not be securely identified from literature. The N2537-40 isolate was found to belong to the genus *Streptomyces*, however, species identification is still unclear. It was hypothesised that the N2537-40 isolate produces a novel lipopeptide due to methyl group differences between molecular species and the absence of compounds in literature with similar M_r values. The antimicrobial compound from this isolate will be subjected to production, purification and characterisation in future studies. Novel antimicrobial compounds purified from the above isolates will be subjected to sequence determination, bio-activity and biophysical characterisation in future studies.

Several soil isolates displayed antimicrobial activity, however, they did not display production of compounds with M_r values within the M_r detection limits of 800 to 3000. The extracts of these isolates will be subjected to further purification in future studies to remove the large amount of inactive compounds resulting in background noise observed in the ESMS

spectra below the M_r value of 800. They will then be screened for the presence of novel low M_r antimicrobial compounds.

Finally, this study has also revealed various shortcomings of using conventional microbiological techniques for the identification of antimicrobial agent producing bacteria from environmental samples. Shortcomings include: inadequate selection of diverse bacterial species, laboriousness and low-throughput screening methodology. In an era where conventional antibiotics are becoming increasingly inefficient, the need for high throughput screening of novel and effective antimicrobial agents escalates. Future studies will therefore be focused at increasing the throughput of antimicrobial peptide screening while decreasing the screening period.

4.6 References

1. Rossello-Mora, R. and Amann, R. (2001) The species concept for prokaryotes. FEMS Microbiol. Rev. **25**, 39-67
2. Hibbing, M. E., Fuqua, C., Parsek, M. R. and Peterson, S. B. (2010) Bacterial competition: surviving and thriving in the microbial jungle. Nat. Rev. Microbiol. **8**, 15-25
3. Maróti, G., Kereszt, A., Kondorosi, É and Mergaert, P. (2011) Natural roles of antimicrobial peptides in microbes, plants and animals. Res. Microbiol. **162**, 363-374
4. Riley, M. A. and Gordon, D. M. (1999) The ecological role of bacteriocins in bacterial competition. Trends Microbiol. **7**, 129-133
5. Dubos, R. J. (1939) Studies on a bactericidal agent extracted from a soil *Bacillus*: I. preparation of the agent. its activity *in vitro*. J. Exp. Med. **70**, 1-10
6. Yang, S. C., Lin, C. H., Sung, C. T. and Fang, J. Y. (2014) Antibacterial activities of bacteriocins: application in foods and pharmaceuticals. Front. Microbiol. **5**, 241-250
7. Hancock, R. E. W. and Sahl, H. G. (2006) Antimicrobial and host-defense peptides as new anti-infective therapeutic strategies. Nat. Biotechnol. **24**, 1551-1557
8. Hancock, R. E. W. (2000) Cationic antimicrobial peptides: towards clinical applications. Expert Opin. Investig. Drugs. **9**, 1723-1729
9. Baltz, R. H. (2008) Renaissance in antibacterial discovery from actinomycetes. Curr. Opin. Pharmacol. **8**, 557-563
10. McCaig, A. E., Grayston, S. J., Prosser, J. I. and Glover, L. A. (2001) Impact of cultivation on characterisation of species composition of soil bacterial communities. FEMS Microbiol. Ecol. **35**, 37-48

11. Rinke, C., Schwientek, P., Sczyrba, A., Ivanova, N. N., Anderson, I. J., Cheng, J. F., Darling, A., Malfatti, S., Swan, B. K., Gies, E. A., Dodsworth, J. A., Hedlund, B. P., Tsiamis, G., Sievert, S. M., Liu, W. T., Eisen, J. A., Hallam, S. J., Kyrpides, N. C., Stepanauskas, R., Rubin, E. M., Hugenholtz, P. and Woyke, T. (2013) Insights into the phylogeny and coding potential of microbial dark matter. Nature. **499**, 431-437

12. Daniel, R. (2004) The soil metagenome-a rich resource for the discovery of novel natural products. Curr. Opin. Biotechnol. **15**, 199-204

13. Travers, R. S., Martin, P. A. and Reichelderfer, C. F. (1987) Selective process for efficient isolation of soil *Bacillus* spp. Appl. Environ. Microbiol. **53**, 1263-1266

14. Tagg, J. R., Read, R. S. D. and McGiven, A. R. (1973) Bacteriocin of a group A S*treptococcus*: partial purification and properties. Antimicrob. Agents Chemother. **4**, 214-221

15. Du Toit, E. A. and Rautenbach, M. (2000) A sensitive standardised micro-gel well diffusion assay for the determination of antimicrobial activity. J. Microbiol. Methods. **42**, 159-165

16. Sousa, A. M., Machado, I., Nicolau, A. and Pereira, M. O. (2013) Improvements on colony morphology identification towards bacterial profiling. J. Microbiol. Methods. **95**, 327-335

17. Braga, P. A. C., Tata, A., Goncalves dos Santos, V., Barreiro, J. R., Schwab, N. V., Veiga dos Santos, M., Eberlin, M. N. and Ferreira, C. R. (2013) Bacterial identification: from the agar plate to the mass spectrometer. RSC Adv. **3**, 994-1008

18. Ben-Jacob, E., Shmueli, H., Shochet, O. and Tenenbaum, A. (1992) Adaptive self-organization during growth of bacterial colonies. Phys. A. **187**, 378-424

19. Tang, X., Thibault, P. and Boyd, R. K. (1992) Characterisation of the tyrocidine and gramicidin fractions of the tyrothricin complex from *Bacillus brevis* using liquid chromatography and mass spectrometry. Int. J. Mass Spectrom. Ion Processes. **122**, 153-179

20. Pathak, K. V. and Keharia, H. (2013) Identification of surfactins and iturins produced by potent fungal antagonist, *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) tree using mass spectrometry. 3. Biotech. **4**, 283-295

21. Maget-Dana, R. and Peypoux, F. (1994) Iturins, a special class of pore-forming lipopeptides: biological and physicochemical properties. Toxicology. **87**, 151-174

22. Peypoux, F., Besson, F., Michel, G., Delcambe, L. and Das, B. C. (1978) Structure de l'iturine C de *Bacillus subtilis*. Tetrahedron. **34**, 1147-1152

23. Quentin, M. J., Besson, F., Peypoux, F. and Michel, G. (1982) Action of peptidolipidic antibiotics of the iturin group on erythrocytes: effect of some lipids on hemolysis. Biochim. Biophys. Acta. **684**, 207-211

24. Peypoux, F., Guinand, M., Michel, G., Delcambe, L., Das, B. C., Varenne, P. and Lederer, E. (1973) Isolement de l'acide 3-amino 12-methyl tetradecanoique et de l'acide 3-amino 12-methyl tridecanoique A partir de l'iturine, antibiotique de *Bacillus subtilis*. Tetrahedron. **29**, 3455-3459

25. Peypoux, F., Besson, F., Michel, G. and Delcambe, L. (1979) Preparation and antibacterial activity upon *Micrococcus luteus* of derivatives of iturin A, mycosubtilin and bacillomycin L, antibiotics from *Bacillus subtilis*. J. Antibiot. **32**, 136-140

26. Besson, F., Peypoux, F., Michel, G. and Delcambe, L. (1976) Characterization of iturin A in antibiotics from various strains of *Bacillus subtilis*. J. Antibiot. **29**, 1043-1049

27. Besson, F. and Michel, G. (1987) Isolation and characterization of new iturins: iturin D and iturin E. J. Antibiot. **40**, 437-442

28. Romero, D., de Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J. W., Arrebola, E., Cazorla, F. M., Kuipers, O. P., Paquot, M. and Perez-Garcia, A. (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant-Microbe Interact. **20**, 430-440

29. Morikawa, M., Daido, H., Takao, T., Murata, S., Shimonishi, Y. and Imanaka, T. (1993) A new lipopeptide biosurfactant produced by *Arthrobacter* sp. strain MIS38. J. Bacteriol. **175**, 6459-6466

30. Wu, X., Ballard, J. and Jiang, Y. W. (2005) Structure and biosynthesis of the BT peptide antibiotic from *Brevibacillus texasporus*. Appl. Environ. Microbiol. **71**, 8519-8530

31. Barsby, T., Warabi, K., Sørensen, D., Zimmerman, W. T., Kelly, M. T. and Andersen, R. J. (2006) The bogorol family of antibiotics: template-based structure elucidation and a new approach to positioning enantiomeric pairs of amino acids. J. Org. Chem. **71**, 6031-6037

4.7 Addendum A

During the isolation of bacterial cultures from a soil sample discussed in Chapter 4, 509 isolates were selected due to colony morphology differences. Only 15 of these isolates were observed to possess antimicrobial activity towards the Gram-positive indicator organism *M. luteus*. Subsequently, the isolates were subjected to organic solvent extraction followed by direct electrospray mass spectrometry (ESMS) analysis to determine whether the isolates produce low M_r compounds resembling antimicrobial peptides from literature. During the experiments sterile TSB agar was used as a negative control. The resulting TSB agar spectra exhibited large background noise between the M_r values of 300 to 800 (Figure A.1). Any compounds produced by the isolates within the M_r range of 300 to 800 could therefore not be distinguished. Several peaks in the TSB agar spectra were also observed between the M_r values of 1120 and 1165 (Figure A.1). These peaks were however absent when the isolate produced compounds with a M_r greater than 800 and were therefore not considered as background noise.

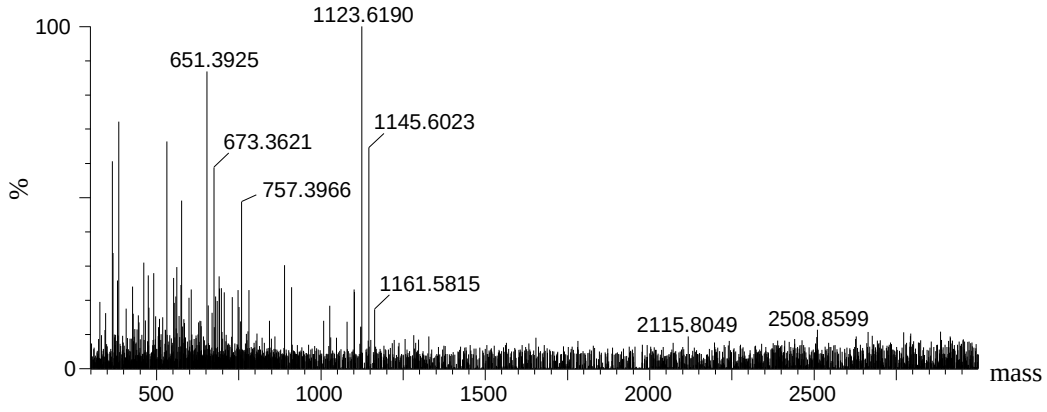


Figure A.1. ESMS spectra from a M_r of 300 to a M_r 3000 following MaxEnt analysis of sterile TSB agar medium, which was used as the negative control during low M_r profile analysis of compounds produced by antimicrobial active soil isolates described in Chapter 4

Three of the isolates namely; N2537-40, N8037-14 and T8037-7, were discussed in Chapter 4 and selected for further investigation. The isolates were selected based on their desirable antimicrobial activity and low M_r ESMS spectra, which was indicative of probable

antimicrobial peptide production. ESMS analysis revealed that the isolates, L2537-30, N2537-15 and N2537-40, produced identical low M_r compounds (Figure A.2 and Figure 4.5 in Chapter 4). However, higher ESMS signal intensities of the identical compounds were observed in the N2537-40 isolate ESMS spectra, which may relate to enhanced antimicrobial production. The L2537-30 and N2537-15 isolates were therefore not discussed in Chapter 4. The remaining ten isolates did not produce any unique low M_r compounds in the 800 to 3000 M_r range, (Figure A.3 and Figure A.4). The absence of low M_r compounds could be either due to the extraction procedure or the production of molecules with a M_r below 800. Future investigations would include a purification step prior to ESMS analysis, to remove compounds leading to background noise, so as to identify compounds produced by the isolates with a M_r below 800.

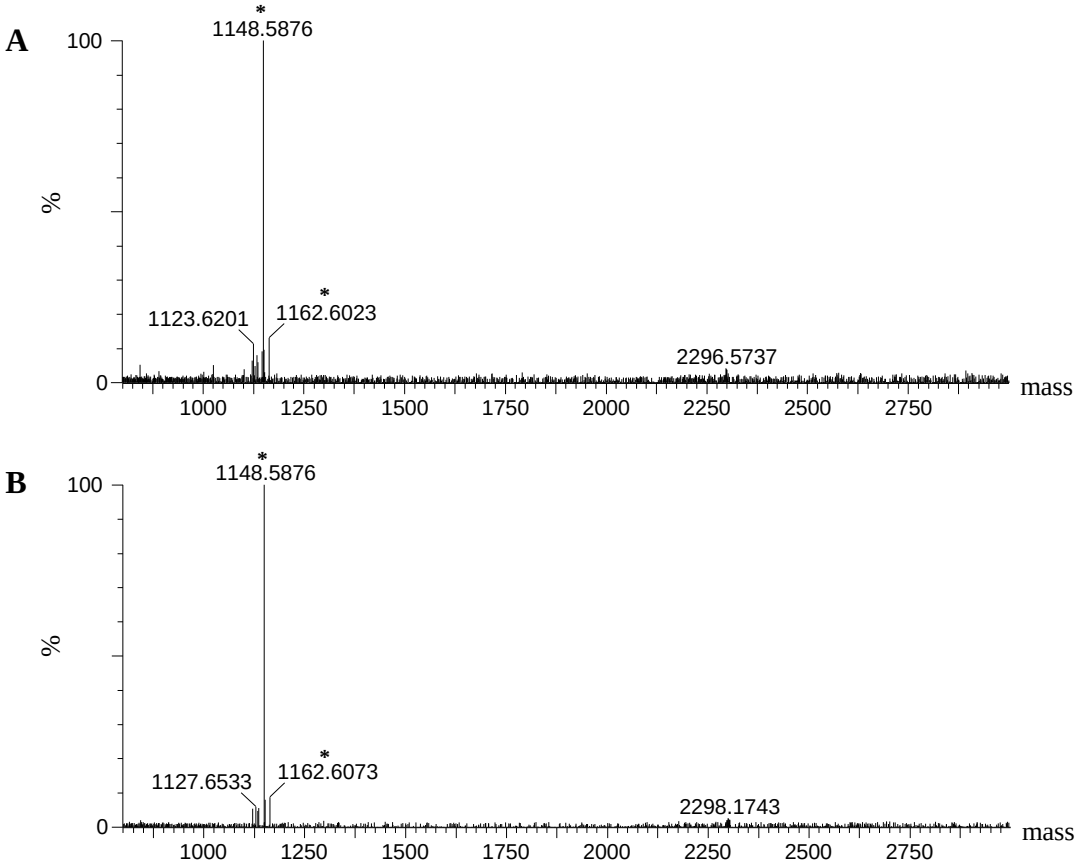


Figure A.2. ESMS spectra analysed by the MaxEnt algorithm displaying M_r values between 800 and 3000 of compounds produced by the soil isolate (A) N2537-15 and (B) L2537-30. These isolates displayed the production of compounds with similar M_r values to that of the N2537-40 isolate, described in Chapter 4, indicated by *.

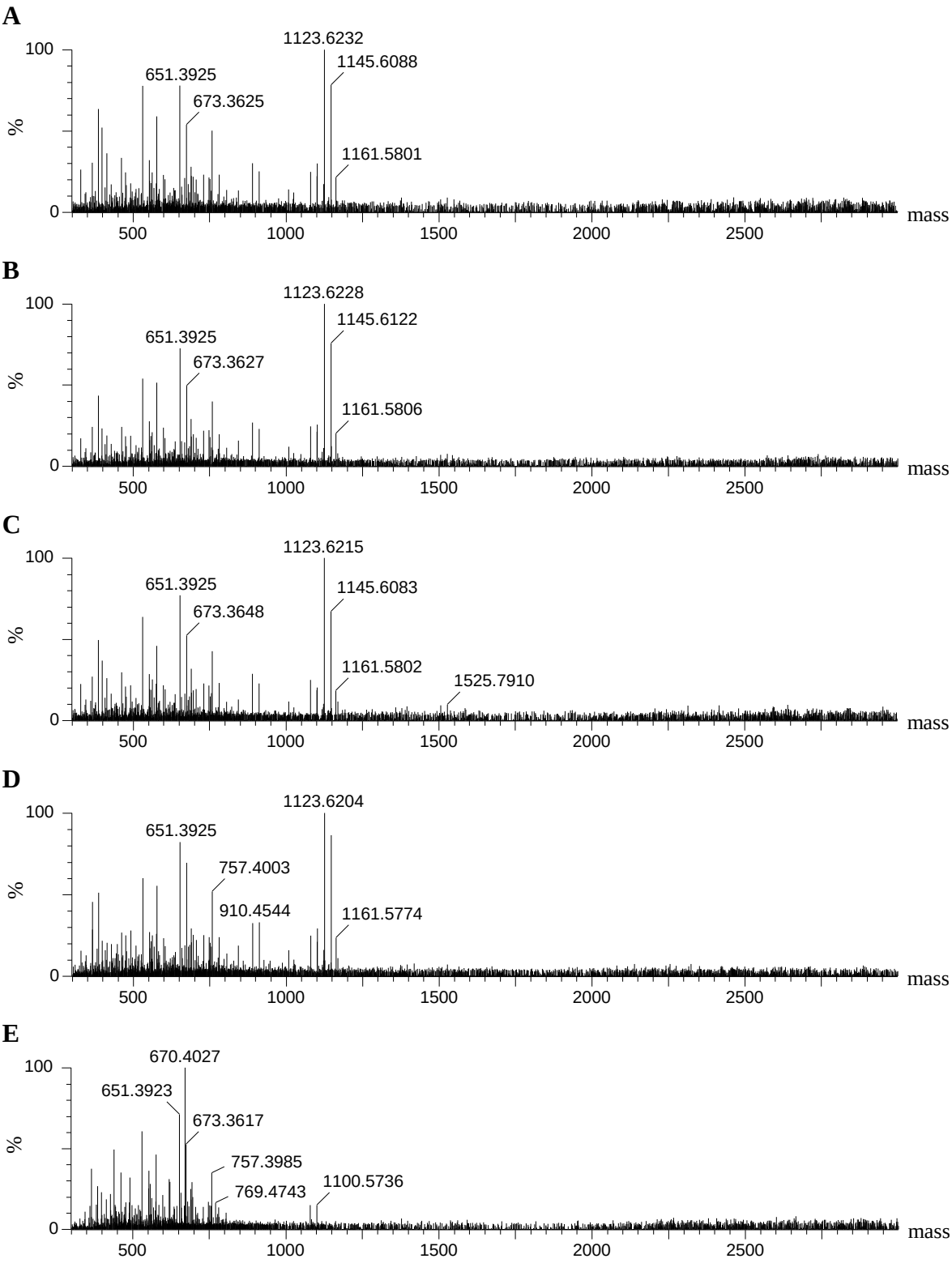


Figure A.3. ESMS analysis of the soil isolates (A) L2525-30, (B) L2525-31, (C) L8025-22, (D) N2525-7 and (E) N2537-3 displaying spectra between the M_r values of 300 to 3000 following MaxEnt analysis.

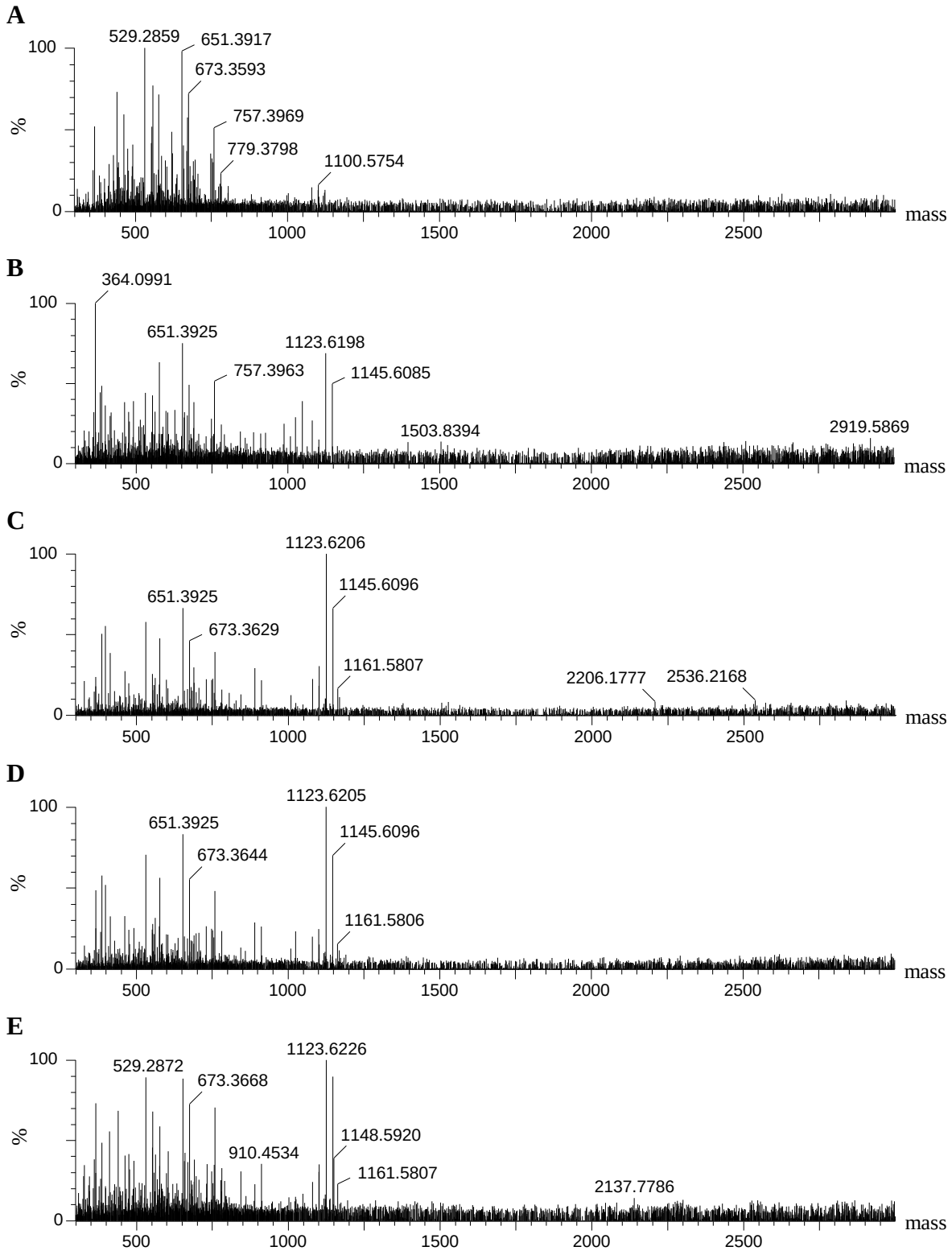


Figure A.4. ESMS analysis of the soil isolates (A) N2537-6, (B) N2537-48, (C) N8025-5, (D) T2525-6 and (E) T2537-10 displaying spectra between the M_r values of 300 to 3000 following MaxEnt analysis.

Chapter 5
Summary, conclusions and future studies

5.1 Introduction

The major goal of this study was to isolate novel antimicrobial peptides from environmental soil samples to aid in the discovery of new and effective antibiotics. We first focused on the isolation and characterisation of antimicrobial peptides from a commercial soil additive. The decreased diversity of the additive, compared to environmental soil samples, simplified the validation of our screening and characterisation methodology. Through the screening process, morphologically different bacterial isolates were selected that showed antibacterial activity against a Gram-positive indicator organism, *Micrococcus luteus*. Antimicrobial peptides were then extracted, purified and identified from bacterial isolates that were selected. Subsequently, the antimicrobial activity, haemolytic activity and mode of action of the antimicrobial peptides were studied. Validated screening methodology was then utilised to screen for bacteria isolated from an environmental soil sample that possibly produce novel antimicrobial peptides.

5.2 Experimental conclusions and future recommendations

In Chapter 2, bacterial isolates were obtained from a commercial soil additive based on differences observed in their colony morphologies. The colony morphology of a bacterial strain may differ depending on environmental factors such as the availability of nutrients and cellular population density [1,2]. A particular bacterial strain can therefore be repeatedly isolated. In Chapter 2 several isolates were hypothesised to be repeatedly isolated due to similarities in antimicrobial activity, haemolytic activity and colony morphology. Although screening based on colony morphological differences is not an accurate method for isolating different bacterial strains, it was used as the initial screening methodology in this dissertation

due to its simplicity and rapidity [3]. Several isolates displayed antimicrobial activity towards the Gram-positive indicator organism *M. luteus*. However, only two isolates, LB.5 and LB.4, were selected for further analysis due to their desirable antimicrobial activity and difference in colony morphology. The LB.5 and LB.4 isolates were identified using a combination of MALDI-MS biotyping and 16S rRNA gene sequencing to be strains of *Bacillus licheniformis* and *Brevibacillus laterosporus* respectively. Due to the lack of strain identifications, the isolates were renamed, *B. licheniformis* LB.5 and *Br. laterosporus* LB.4.

A new ESMS method was developed (Chapter 2) involving the direct analysis of organic extracts obtained from bacterial cultures grown on solid nutrient growth media by direct injection ESMS, to identify the production of low M_r compounds. A MALDI-MS method, comprising of the direct analysis of bacterial colonies without prior organic extraction, was done in parallel with the newly developed ESMS method for the detection of low M_r compound production. The mass spectra of the two methods were compared to establish the more suitable method for the detection of low M_r compounds produced by bacterial cultures. The ESMS method was observed to be more sensitive and accurate and therefore used in further analysis. However, one shortcoming of the ESMS method, observed in Chapter 4, is the presence of significant background noise of compounds between the M_r values of 300 and 800. The origin of the background noise has not yet been determined. However, it was hypothesised to result from growth media and plasticisers in the growth vessels, as different growth vessels displayed variability in background noise (data not shown). Therefore, it is recommended that future investigations should be aimed at determining the suitability of growth vessels and growth media used for this method.

The low M_r ESMS spectra of *B. licheniformis* LB.5 displayed the presence of one highly abundant compound with a M_r of 1421.7529, among several less abundant compounds. The M_r of 1421.7529 was found to be similar to that of the known antimicrobial peptide produced

by *B. licheniformis*, bacitracin A (M_r = 1421.7489) [4,5]. Therefore, we hypothesised that *B. licheniformis* LB.5 produces bacitracin A. In Chapter 3, the antimicrobial compound was purified from cultures of *B. licheniformis* LB.5 and was shown to have a M_r of 1421.7465. The ESMS/MS fragmentation profile of the active compound was also found to be identical to that of commercial bacitracin A. Thus, it was concluded that *B. licheniformis* LB.5 produces the antimicrobial peptide bacitracin A.

The low M_r ESMS spectra of *Br. laterosporus* LB.4 cultures revealed the production of several compounds that may potentially be antimicrobial peptides. The M_r of two compounds (M_r values of 1569.0597 and 1583.0856) were found to closely correlate with that of the antimicrobial peptides analogues produced by *Br. laterosporus* PNG-276, bogorol B (M_r = 1569.0633) and bogorol A (M_r = 1583.0790) [6]. It was therefore hypothesised, due to producing organism and M_r similarities, that *Br. laterosporus* LB.4 produces the antimicrobial peptides bogorol A and bogorol B. The antimicrobial peptide analogues, BT1569 (M_r = 1569.0097) and BT1583 peptide (M_r = 1583.1154) were found from literature to have M_r values similar to that of 1569.0597 and 1583.0856 [7]. However, their M_r difference did not indicate a single amino acid substitution and was outside the mass error range (< 10 ppm). It is therefore unlikely that *Br. laterosporus* LB.4 produces either BT1569 or BT1583 peptide. However, following production and purification, two antimicrobial compounds were found to be produced by *Br. laterosporus* LB.4 (Chapter 3). The M_r values of the compounds were determined to be 1222.6403 and 1272.6167, contradicting the initial hypothesis that *Br. laterosporus* LB.4 produces bogorol A and bogorol B. The hypothesis could, however, not be disproved as bogorol A and bogorol B might not have been observed in this study due to the particular culturing and extraction methodology used. A subsequent review of literature was indicative of probable novelty, as no antimicrobial compounds were found with M_r values similar to 1222.6403 and 1272.6167. The compounds were therefore

preliminarily named LB.4-1223 and LB.4-1273. Both LB.4-1223 and LB.4-1273 were determined to be peptides comprising of the amino acids: Tyr, Phe, Pro, Leu/Ile, Met, Val and Asn. The precise amino acid sequence of the peptides, however, could not be determined as of yet. Future studies will be aimed at the determination of the primary amino acid sequence of both the peptides to confirm their novelty. Primary sequences will allow future investigations into secondary and tertiary peptide structure which, in turn, will aid structural activity relationship studies.

The antimicrobial peptide tryptocidine C was also purified from cultures of *Brevibacillus parabrevis* in chapter 2. The antimicrobial and haemolytic activity of tryptocidine C has been previously determined. Furthermore, it has been shown to exert is mode of action via the disruption of the cellular membrane [8-10]. Therefore, it was used as a comparative, positive control during activity assays and mode of action studies.

Activity studies towards *M. luteus* and erythrocytes indicated that bacitracin A exhibited no significant haemolytic activity and much greater antimicrobial activity compared to the other peptides, supporting its use as pharmaceutical antibiotic [11]. Tryptocidine C, LB.4-1223 and LB.4-1273 exhibited both antimicrobial and haemolytic activity. LB.4-1273 displayed antimicrobial activity comparable to that of tryptocidine C, whereas less antimicrobial activity was observed for LB.4-1223. The haemolytic activity of both LB.4-1223 and LB.4-1273 was comparable to that of tryptocidine C. The decreased antimicrobial activity observed for LB.4-1223 may have been due to the formation of an emulsion in the working solvent rather than going into a solution. The formation of an emulsion in the working solvent, was also observed for LB.4-1273. Therefore, the antimicrobial and haemolytic activity of both LB.4-1223 and LB.4-1273 may be underestimated due to precipitation of the peptide emulsion. Further investigation will be done with appropriate solvents so as to fully dissolve the peptides while not affecting the activity assays. The study only provided information on

the antimicrobial activity of the peptides towards the Gram-positive bacterium, *M. luetus*. Future studies will therefore be focused at determining the antimicrobial activity of tryptocidine C, LB.4-1223 and LB.4-1273 towards a broad range of clinically important bacterial, fungal and protozoan pathogens. Bacitracin A has been clinically used for many years and its range of antimicrobial activity extensively studied [11]. Therefore, no further analysis on the range of bacitracin's antimicrobial activity is needed.

In Chapter 3, ^{31}P solid state NMR analysis on macroscopically orientated lipid bilayers was utilised to assist in the elucidation of the mode of action of tryptocidine C and bacitracin A. Both bacitracin A and tryptocidine C were shown to interact with macroscopically orientated lipid bilayers that mimic eukaryotic, Gram-positive and Gram-negative bacterial cellular membranes respectively. ^{31}P solid state NMR spectra indicated that the interactions resulted in disorientation of the lipid bilayer phospholipid head groups as well as a phase transition shift towards a more liquid state, thus supporting tryptocidine C's membrane-mediated mode of action as observed by previous investigators [8-10]. Bacitracin A has previously been shown to mainly exert its antibacterial activity by inhibiting peptidoglycan synthesis [12]. However, Storm and Strominger [13] hypothesised that bacitracin retains a membrane-mediated mode of action as well, due to their observations of protoplast membrane disruption in the presence of bacitracin. No further studies, however, were found to investigate the membrane-mediated mode of action of bacitracin. Therefore, to our knowledge this study provided one of the first insights into the interactions between bacitracin A and cellular membranes.

Electrophysiological experiments (Chapter 3), in contrast to ^{31}P solid state NMR experiments, showed pore formation in membranes only when tryptocidine C was present. In lipid bilayers mimicking eukaryotic and Gram-positive bacterial cellular membranes, the pores were more defined with a narrow pore size range. Together with the disorientation

observed with ^{31}P solid state NMR this might indicate a vertical insertion of the peptides in the membrane, thus forming channels. However, a wide pore size range was observed for lipid bilayers mimicking those of Gram-negative bacterial cell membranes. Furthermore, the majority of pores in the Gram-negative bacterial cellular membrane lipid bilayer model were larger than the pores observed for eukaryotic and Gram-positive bacterial cellular membrane models. Together with the disorientation observed with ^{31}P solid state NMR it can be speculated that tryptocidine C orientates horizontally within the membrane which in turn might lead to membrane leakage. Conversely, it was found that bacitracin did not form pores in the membranes. This suggests that the interactions observed between bacitracin A and the membrane models, during ^{31}P solid state NMR studies, may only aid in transmembrane transportation of the peptide towards its intracellular target, resulting in peptidoglycan synthesis inhibition.

This study only provided initial insights into the membrane-mediated mode of action of both tryptocidine C and bacitracin. Unfortunately, significant amounts of the newly discovered LB.4-1223 and LB.4-1273 peptides could not be purified during this study for biophysical analysis. These peptides will therefore be purified and subjected to biophysical analysis in subsequent studies. Further investigation also needs to be aimed at deciphering the exact mechanism by which tryptocidine C and bacitracin A interact and possibly insert into cellular membranes; to provide a clear understanding of the peptides' membrane-mediated mode of action and/or role in antimicrobial activity. Future mode of action studies would greatly benefit from isotopically labelled peptides which can be subjected to detailed solid state NMR analysis in combination with lipid bilayers. Thus, one main focus would be the production or synthesis of isotopically labelled peptides.

In Chapter 4, a soil sample collected in Stellenbosch, South Africa, was screened for antimicrobial producing bacteria using the methodology validated in Chapter 2. Only three

bacterial isolates from a total of 507 initially isolated were found to produce compounds with desirable M_r values (M_r values between 800 and 3000) and antimicrobial activity namely: T8037-7, N8037-14 and N2537-40. The isolate T8037-7, identified to be a species of *Bacillus*, was found to produce a compound with a M_r similar to Iturin C2, a member of the iturin group of antimicrobial peptides [14]. Iturin C2 was, however, disregarded as a candidate responsible for the antimicrobial activity of the T8037-7 isolate, due to conflicting bio-activity and the absence of the distinctive iturin lipopeptide complex during ESMS analysis [15-17]. Further investigation of the T8037-7 isolate will thus be focused on antimicrobial active compound(s) and *Bacillus* species identification. Isolate N8037-14 was identified to be a strain of *Br. laterosporus*. The low M_r ESMS spectra obtained from a culture of the N8037-14 isolate indicated the production of two compounds with almost identical M_r values to bogorol A and bogorol B, produced by *Br. laterosporus* PNG-276 [6]. These compounds were also similar to compounds produced by *Br. laterosporus* LB.4 that was isolated in Chapter 2. Furthermore, a major compound produced by the N8037-14 isolate with a M_r of 1353.8691 could not be clearly identified from literature, suggesting probable novelty. No concrete conclusions could therefore be reached regarding the antimicrobial compound(s) produced by the N8037-14 isolate. The last isolate, N2537-40, was identified to be a *Streptomyces* spp., however, methodology used during this study could not provide species identification. Species identification in future investigations would have to be done using different combinations of primers. One major compound was produced by the N2537-40 isolate that did not possess a M_r value similar to antimicrobial compounds found in literature. Major compound and minor compounds produced by the N2537-40 isolate differed from one another by a M_r of 14.01, which can be ascribed to a methyl group difference. Methyl group differences may be indicative of the biosynthesis of a varying acyl chain

lipopeptide. It was therefore hypothesised that the N2537-40 isolate produces a potentially novel antimicrobial lipopeptide.

Investigations completed to date on the T8037-7, N8037-14 and N2537-40 isolates only provided probable identification of antimicrobial compounds produced. Future studies would have to be aimed at purifying and identifying antimicrobial compounds from the respective isolate cultures using methodology described in Chapter 3. Novel antimicrobial compounds found from these isolates would also be subjected to antimicrobial activity, toxicity and biophysical analysis to aid in the determination of commercial viability.

Finally, this study has also revealed various shortcomings of using conventional microbiological techniques for the identification of antimicrobial agents from natural environments. Shortcomings include: inadequate selection of diverse bacterial species, laboriousness and low throughput screening methodology. In an era where conventional antibiotics are becoming increasingly inefficient due to antimicrobial resistance, the need for high throughput screening methodology to identify novel and effective antimicrobial agents is augmented. Several high throughput screening methodologies based on genomic analysis and *de novo* synthesis for the discovery of novel antibiotics are currently employed [18]. No significant advances, however, have been made towards directly prospecting naturally produced antimicrobials from environmental isolates, which is said to be the source of the most efficient antibiotics [18,19]. Thus, a main focus of future investigations would be the development of a medium and/or high-throughput method to screen for antimicrobial producing organisms and their respective antimicrobial compounds.

We have already developed a hypothetical method for medium-throughput screening (Figure 5.1) and have preliminary validated several steps. The method can be divided into three parts. In the first part (Figure 5.1 A), environmental samples are suspended and then diluted to theoretically add one cell per well in 96-well plates containing various complex growth

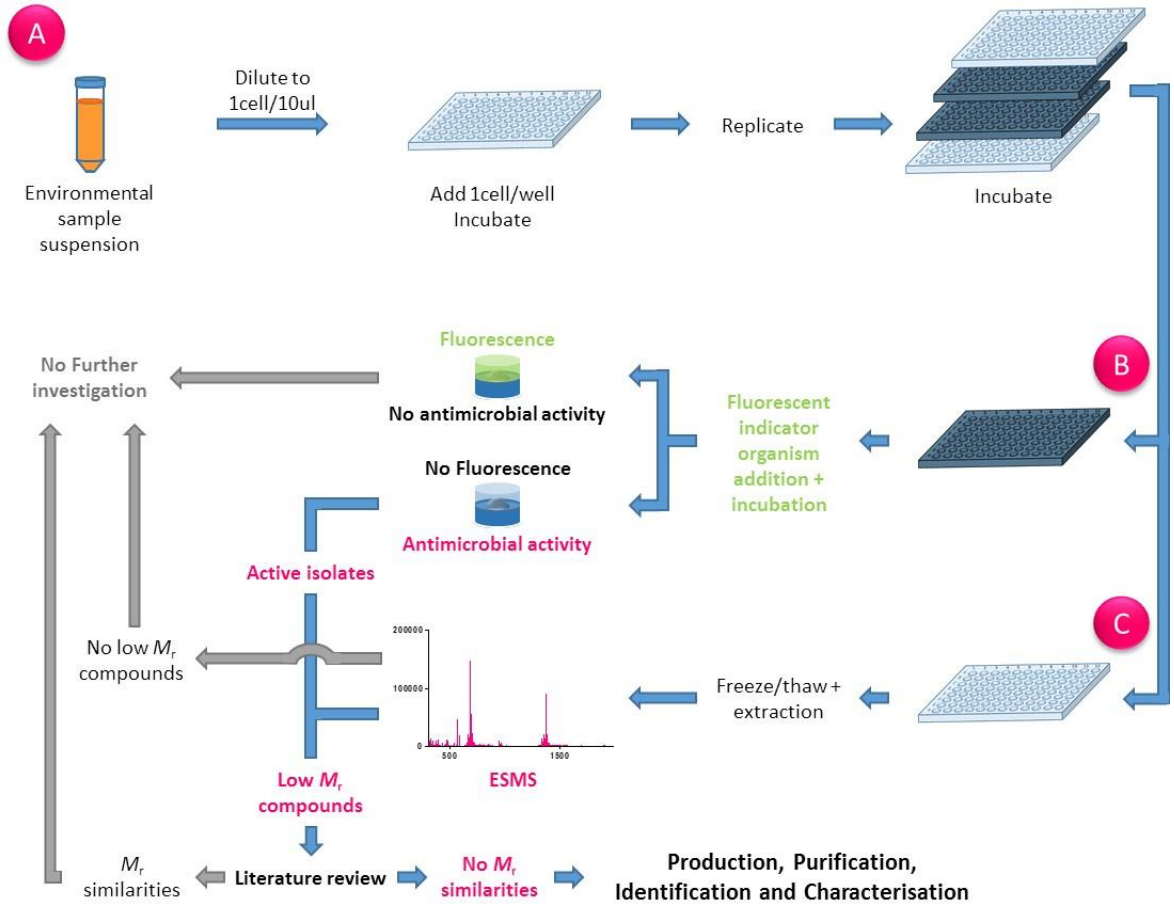


Figure 5.1. Hypothesised medium throughput method for the screening of environmental samples to identify novel antimicrobials. The method is divided into three parts namely: (A) inoculation, isolation and incubation, (B) antimicrobial activity detection and (C) low molecular mass ESMS fingerprint identification. Only isolates displaying antimicrobial activity and the production of unique low M_r compounds would be subjected to further investigation.

media. After incubation, the plates are replicated onto several separate 96-well plates, which includes a control plate as well as plates used during parts two and three. Part two of the method (Figure 5.1 B) entails the detection of the antimicrobial activity of the isolates towards various indicator organisms. Indicator organisms that expresses green fluorescent protein (GFP) are simultaneously cultured with the isolates and fluorescence used as indication of indicator organism growth inhibition. This simultaneous culturing method has already been validated and is currently in the process of optimisation. The last part (Figure 5.1 C) makes use of the newly developed ESMS method, described in Chapter 2, to detect the production of low M_r compounds so as to exclude previously discovered antimicrobials.

Thus, only antimicrobially active isolates producing unique low M_r compounds would be subjected to further investigation including: production, active compound purification, bacterial and compound identification and lastly compound characterisation. Future studies will be aimed at validating, refining and optimising the method as a whole, so as to aid in the efficient prospecting for naturally produced antimicrobial peptides and other antimicrobial agents in an attempt to pre-empt the complete resistance of pathogens to current and conventional antibiotics.

5.3 References

1. Sousa, A. M., Machado, I., Nicolau, A. and Pereira, M. O. (2013) Improvements on colony morphology identification towards bacterial profiling. J. Microbiol. Methods. **95**, 327-335
2. Ben-Jacob, E., Shmueli, H., Shochet, O. and Tenenbaum, A. (1992) Adaptive self-organization during growth of bacterial colonies. Phys. A. **187**, 378-424
3. Braga, P. A. C., Tata, A., Goncalves dos Santos, V., Barreiro, J. R., Schwab, N. V., Veiga dos Santos, M., Eberlin, M. N. and Ferreira, C. R. (2013) Bacterial identification: from the agar plate to the mass spectrometer. RSC Adv. **3**, 994-1008
4. Johnson, B. A., Anker, H. and Meleney, F. L. (1945) Bacitracin: a new antibiotic produced by a member of the *B. subtilis* group. Science. **102**, 376-377
5. Siegel, M. M., Huang, J., Lin, B., Tsao, R. and Edmonds, C. G. (1994) Structures of bacitracin A and isolated congeners: sequencing of cyclic peptides with blocked linear side chains by electrospray ionization mass spectrometry. Biol. Mass Spectrom. **23**, 186-204
6. Barsby, T., Warabi, K., Sørensen, D., Zimmerman, W. T., Kelly, M. T. and Andersen, R. J. (2006) The bogorol family of antibiotics: template-based structure elucidation and a new approach to positioning enantiomeric pairs of amino acids. J. Org. Chem. **71**, 6031-6037
7. Wu, X., Ballard, J. and Jiang, Y. W. (2005) Structure and biosynthesis of the BT peptide antibiotic from *Brevibacillus texasporus*. Appl. Environ. Microbiol. **71**, 8519-8530
8. Leussa, A. N. (2014) Characterisation of small cyclic peptides with antimalarial and antilisterial activity. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/86161
9. Spathelf, B. M. (2010) Qualitative structure-actvity relationships of the major tyrocidines, cyclic decapeptides from *Bacillus aneurinolyticus*. Stellenbosch University, Department of Biochemistry, Stellenbosch, South Africa. **PhD. Thesis,** http://hdl.handle.net/10019.1/4001

10. Aranda, F. J. and de Kruijff, B. (1988) Interrelationships between tyrocidine and gramicidin A′ in their interaction with phospholipids in model membranes. Biochim. Biophys. Acta. **937**, 195-203

11. Werner, A. H. and Russell, A. D. (1999) Mupirocin, fusidic acid and bacitracin: activity, action and clinical uses of three topical antibiotics. Vet. Dermatol. **10**, 225-240

12. Stone, K. J. and Strominger, J. L. (1971) Mechanism of action of bacitracin: complexation with metal ion and C_{55}-isoprenyl pyrophosphate. Proc. Natl. Acad. Sci. U. S. A. **68**, 3223-3227

13. Storm, D. R. and Strominger, J. L. (1974) Binding of bacitracin to cells and protoplasts of *Micrococcus lysodeikticus*. J. Biol. Chem. **249**, 1823-1827

14. Pathak, K. V. and Keharia, H. (2013) Identification of surfactins and iturins produced by potent fungal antagonist, *Bacillus subtilis* K1 isolated from aerial roots of banyan (*Ficus benghalensis*) tree using mass spectrometry. 3. Biotech. **4**, 283-295

15. Peypoux, F., Besson, F., Michel, G., Delcambe, L. and Das, B. C. (1978) Structure de l'iturine C de *Bacillus subtilis*. Tetrahedron. **34**, 1147-1152

16. Quentin, M. J., Besson, F., Peypoux, F. and Michel, G. (1982) Action of peptidolipidic antibiotics of the iturin group on erythrocytes: effect of some lipids on hemolysis. Biochim. Biophys. Acta. **684**, 207-211

17. Romero, D., de Vicente, A., Rakotoaly, R. H., Dufour, S. E., Veening, J. W., Arrebola, E., Cazorla, F. M., Kuipers, O. P., Paquot, M. and Perez-Garcia, A. (2007) The iturin and fengycin families of lipopeptides are key factors in antagonism of *Bacillus subtilis* toward *Podosphaera fusca*. Mol. Plant-Microbe Interact. **20**, 430-440

18. Lewis, K. (2013) Platforms for antibiotic discovery. Nat. Rev. Drug. Discov. **12**, 371-387

19. Clardy, J., Fischbach, M. A. and Walsh, C. T. (2006) New antibiotics from bacterial natural products. Nat. Biotechnol. **24**, 1541-1550